SOME QUANTITATIVE STUDIES ON REFECTION IN THE RAT [1]

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TWO FIGURES

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The general theories concerning the nature of refection have been summarized by Browning ('31) and by Bliss ('36). Opinion in America concerning the phenomenon has been divided. Mendel and Vickery ('29) failed to obtain it on standard rations and raised the question of a possible role of residual vitamin B in the diet itself. On the other hand, its occurrence has been reported by Kon ('31) and Lepkovsky[2] from Professor Evans' laboratory; by Parsons, Kelly and Hussemann ('33), and Parsons and Kelly ('35); and by Bliss ('36). Each of these interpreted this occasional growth of rats on vitamin B-low rations in agreement with the general

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[2] It was learned by personal communication from Doctor Lepkovsky that some observations on this subject were given by him in a verbal report before a meeting of the American Chemical Society.

hypothesis first formulated by Fridericia ('26). In contrast with these, on the other hand, the assumption was made by Whipple and Church ('35) that the phenomenon is essentially dependent on the small amount of non-extractable fat in raw starch which is made available to the animal when the starch granule is ruptured in the digestive tract; this theory grew out of their observation that the 'content' (i.e., concentration) of the anti-beriberi factor in the feces of rats on vitamin B-low rations in their experiments was directly correlated with the amount of lard in the diet.

EXPERIMENTAL

First occurrence of refection in this laboratory

Refection was first observed in 1931 in this laboratory during the progress of a rough assay of some foodstuffs for their vitamin G content. Potato starch was tested both as a crude preparation and as a commercial sample by substituting it for 35% of cornstarch when growth had ceased after 2 weeks on the following ration; purified casein 18, Osborne and Mendel's[3] salt mixture 4, rice polishings 6, cod liver oil 2, sucrose 35 and cooked cornstarch 35%. There was a slow gradual gain after which growth again ceased. Most of the group was discarded at this time but not before a part of them had made a sudden unexpected gain. Of these, three were retained because a few days before the upward turn occurred, large white feces had been noticed on the papers under the cages. Average gains of 2.5 gm. per day were made by these rats for several weeks after which the rice polishings were removed from the ration of one rat and reduced to 2% in the ration of another; the former rat lost weight steadily and was discarded, but the latter continued to gain until it weighed 332 gm. At this point cooked potato starch was substituted for the raw, whereupon the rat lost weight rapidly and died. The condition was thereafter produced in a succession of animals on the diet on which this rat had thrived: casein 18, salt mixture 4, rice polishings 2, cod liver oil 2, sucrose 24 and raw potato starch 50. This will be referred to as ration 1.

[3] Osborne and Mendel ('19).

Characteristics of the condition secured on rations containing rat potato starch

The feces of the presumably refected rats were bulky with occluded air spaces and were loaded with undigested starch grains clearly visible under the microscope.[4] The color ranged from creamy white to brownish tan; the degree of whiteness was not well correlated either with the concentration of vitamin B in the refected feces or with the rapidity of the rat's growth; in fact, after the condition was established, large brownish-tan feces seemed to be as good an indication of its persistence as were strikingly white feces. It is conceivable that the latter are rather an indication of the condition which presumably initiates refection, i.e., the passage of a large amount of undigested starch granules into the coecum, thus providing a substrate for the multiplication of organisms, but that when these are well established their activity may often result in the splitting of the greater part of the raw starch entering the coecum, thus yielding brown rather than white feces albeit with an accompanying production of a large amount of vitamin B.

Attempts to establish refection on purified rations containing raw potato starch but no other possible source of the vitamin B-complex were not successful, contrary to the experience of others; although white feces appeared as early on these purified rations as on those containing rice polishings, the animals did not thrive; only when small amounts of some substance such as rice polishings or autoclaved yeast were included in the ration was growth obtained. Preformed vitamin B seemed not to be responsible for the effects of these additions inasmuch as the yeast had received thorough heat treatment. Young rats at weaning, fed a standard vitamin B-low ration containing 6% of autoclaved yeast as a source of the vitamin B complex, until the body weights declined, began to grow rapidly and to show other signs of refection at varying intervals after 50% of potato starch was substituted for an equal amount of sucrose in the ration and the

[4] Samples of these refected feces were exhibited in a demonstration given at the meeting of the American Society of Biological Chemists at Cincinnati, 1933.

feces eliminated were made available to the rats. Reproduction was successful in the first generation of these rats but not in the second. Assays showed that the feces contained considerable, although variable, amounts of vitamin B.

The reason for the lack of digestion of raw potato starch eliminated in feces containing diastase, a phenomenon recorded by various observers, is not known. In a few of the present experiments the progress of the digestion of the potato starch with saliva and malt diastase was followed by observations under the microscope. After 72 to 144 hours many of the larger grains were fissured or broken but the smaller grains were apparently untouched, in harmony with the observations of Whymper ('09) on the relative resistance of the latter to the action of various agents. No qualitative difference was noted between the behavior of the starch of the ration and that of the grains remaining undigested in the feces. This is in harmony with the results of Bliss ('36). The experiment suggested, however, that the peculiar resistance to digestion which the potato starch was observed to acquire by passage through the digestive tracts of the refected rats in Fridericia's laboratory (Fridericia and associates, '27–'28, and Nathan, '34) may have been due to a disappearance of most of the large starch grains in the digestive tracts of the rats either by the action of the digestive juices or of organisms, thus leaving the starch of the feces made up much more largely of small resistant grains than was the original starch of the ration.

Meteorism was intense in the rats on raw potato starch rations, even causing the death of animals that had been on the ration for some time and were gaining rapidly.

Characteristics of the condition secured on rations containing gelatinized potato starch

It was desirable to test the performance of rats on ration 1 when the potato starch of the ration had been subjected to sufficient moist heat to render the starch grains readily digestible thus presumably reducing the amount of food material reaching the coecum, but at the same time avoiding any

detectable destruction of vitamin B. Bare gelatinization of the starch seemed to answer these requirements; this was accomplished by quickly combining a cold water suspension of the starch with sufficient hot water to result either in a temperature of 63 to 65° which was held for 5 minutes, or a temperature of 70 to 73° which was followed by as rapid cooling of the paste as possible in thin layers. These methods are comparable with that of Bliss ('36). As in the experiments with raw starch, the technic was also followed here of feeding 3% of refected feces for 9 days. Of the group of forty rats started on this ration, none gave those evidences of refection that had been observed in the group on raw starch in regard to growth or size and color of feces. The individual gain in weight during the first 3 weeks averaged 17 gm. for the group. The feces were uniformly small, hard and very dark. Many of the group were discarded when growth ceased at some time before the end of the third to fifth week. Of the remainder, fifteen rats were observed to develop quite definite manifestations of a nutritive disorder, from the twenty-fourth to the forty-third day of the experiment. These manifestations included a huddled posture, sharply bent back, persistence in clutching and clinging to the wires of the cage, insecure gait, and difficulty of balance with a tendency to turn in circles, to hold the head on one side and to fall. Two rats in an extremely serious condition made a swift and apparently complete recovery after injections of Eli Lilly and Company's[5] concentrate of vitamin B_1. Inasmuch as the symptoms were so closely similar to those described for vitamin B_4 deficiency, a preparation of this vitamin, kindly furnished by Dr. C. A. Elvehjem, was fed by mouth to two rats but gave slight or even doubtful benefit. It seems quite possible that the deficiency was multiple but certainly the lack of vitamin B was pronounced.

Evidence will be cited later in more nearly quantitative experiments, that there was no significant destruction of vita-

[5] Thanks are due to Eli Lilly and Company of Indianapolis for furnishing this extract.

min B during gelatinization if, indeed, this vitamin occurred in appreciable concentration in the potato starch used.

Comparison of the vitamin B in the feces of rats on equalized intakes of rations containing either high or low fat and either raw or gelatinized potato starch

Although the performance of rats on vitamin B-low rations containing raw potato starch was clearly superior to that on gelatinized, it seemed to us that one aspect of the problem had not been considered by the objectors to Mendel and Vickery's theory when they argued that all animals would behave alike if the growth in refection were attributable to residual vitamin B in the ration; this aspect was that rats on potato starch rations show wide variations in regard to the amount of potato starch eliminated in their feces, which, as Fridericia has shown may amount to as much as 62% of the weight of the feces on raw starch rations. This fraction of potato starch, not being absorbed and utilized by the body, would presumably involve a corresponding increase in the food intake of these rats in comparison with rats not eliminating starch in their feces and might thus conceivably account for an unequal intake of vitamin B if residues of this vitamin were in fact present in the ration. To test this point, the following experiment was undertaken whereby the effects of making the starch readily digestible and of adding a fat to the ration were separated from the effects of inequalities of food intake by carefully equalizing the latter factor.

Details of the experiment. Two groups of rats weighing about 45 gm. were fed ration 1 containing raw potato starch and gelatinized potato starch, respectively; the rats on the contrasting diets were paired, the food intake of the pair being controlled by the pacemaker and varied from day to day. With the results of this experiment as a guide in regard to the intake of food which it was possible to achieve, other groups of six rats each were started with rigid daily food intakes of 3.5, 4, 4.5 or 6 gm. For these, both the raw and the gelatinized starch rations were varied by the substitution

of 10% of lard for an equal weight of sucrose in the rations of certain groups. Many rats had to be dropped from each group because of failure in food intake, and larger rats were substituted, care being taken to keep the average weights of the various groups approximately equal. In the case of the groups on gelatinized starch, only those on 4 gm. or less daily intake of the ration containing lard included as many as six rats at the end of the period.

The vitamin B-content of the feces eliminated by these donor groups over equal periods of time were tested by feeding them to an equal number of assay rats either as intact individual daily collections or after mixing, pulverizing, and redividing the total 30 days' feces collection of a given donor rat. During a preliminary 9-day period, 3% of refected feces were included in the ration of the donor rats in order to equalize the opportunity for the transmission of the agent of refection to the rats in the various groups in case this precaution should be needed; the collections of feces of the donors were discarded during these 9 days. No difficulty was experienced in securing consumption of the feces doses by the assay groups. Control groups of assay rats were fed the basal ration alone and with the addition of 2 international units of vitamin B per day supplied in weighed portions of a yeast standardized by Dr. E. M. Nelson of the United States Department of Agriculture, Washington, D. C., against the international vitamin B preparation of the Health Committee of the League of Nations.

It was considered essential to assay some representative body tissues of the donor rats for their content of vitamin B to furnish evidence as to whether or not a possibly high content of vitamin B in the feces might represent losses of body stores of this vitamin, or a low content, uncontrolled coprophagy with a storage of the vitamin in the tissues.

The body organs which were assayed were taken from the larger rats in each group; they comprised the liver, kidneys, spleen, heart and lungs. These were removed from the body after the donor rat had been bled from the jugular vein

under anaesthesia and killed. These tissues were then minced with scissors, dried as one preparation from each rat at room temperature before an electric fan and pulverized with a mortar and pestle. In preliminary experiments, doses of 0.7 gm. of such dried preparations were given to assay rats which had been losing weight for 3 to 4 days on the vitamin B-low ration, and the dosage was repeated when the body weights had again decreased to the same point after the gain due to the dosage. In this way, a very good estimate could be made of the most favorable concentration at which to feed the later samples, as a part of the vitamin B-low ration, to bring out comparisons between groups. Thereafter, a composite sample from three donor rats was fed incorporated in the ration of one assay rat in a 15-day test period. This time interval was considered fully adequate in view of the observations of Knott and Schultz ('36) on a 10-day vitamin B assay.

Although none of the rats was observed to practice coprophagy at any time, the harness of Page ('32) was tested but was found to be unsuitable for our purpose. It obviously could not be used for the rats with intense meteorism; even for the others it was discarded since it was necessary to draw it so tightly as to seem to cause severe discomfort and interfere with food intake.

Results. It may be seen in figure 1 that the growth of the donors on any one level of food intake was quite comparable for the most part, but it is notable that growth was somewhat poorer on the raw then on the gelatinized starch ration with an equal calorie intake, possibly due to loss of some of the starch in the feces from the former, resulting in a lower actual intake of energy.

When one examines the growth of the assay rats in figure 2 and table 1 there is seen to be a striking difference between the average vitamin B-content of the feces produced on a ration containing 50% of gelatinized potato starch with 10% of lard (group 1) and that on an equal intake of a comparably fat-rich ration containing raw potato starch (group 2). Some curves of group 2 were equal to those of rats fed 2 international units

of vitamin B daily, whereas no rat in group 1 made any sustained gain. That this difference between groups 1 and 2 is not attributable to an unequal content of performed vitamin B in the ration itself dependent on a destruction of the vitamin during the gelatinization of the potato starch is clearly proven by the fact that the feces of one of the donors on raw potato starch (group 2) were no richer in vitamin B

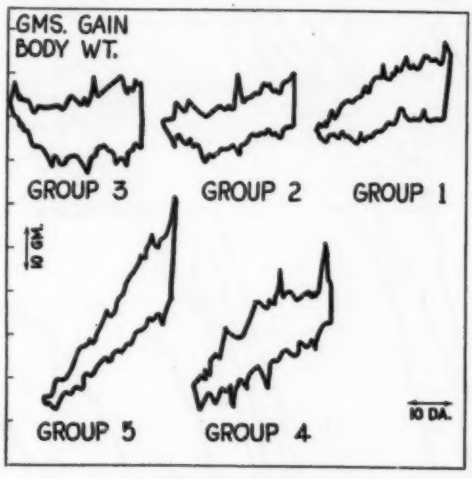

Fig. 1 Solid areas represent combined growth curves of sets of donor rats on the following diets: Group 1, 4 gm. daily intake of ration 1 with gelatinized potato starch + 10% lard. Group 2, 4 gm. daily intake of ration 1 with raw potato starch + 10% lard. Group 3, 4 gm. daily intake of ration 1 with raw potato starch. Group 4, 6 gm. daily intake of ration 1 with raw potato starch + 10% lard. Group 5, 6 gm. daily intake of ration 1 with raw potato starch.

than those of some of the donors on gelatinized. To determine whether or not the failure of this one assay rat was due to an inability to grow, it was fed 0.5 gm. per day of feces from another donor rat; the prompt and vigorous response in growth, represented by the dotted line, gave ample evidence that the previous lack of growth was in fact due to the low content of vitamin B in the first donor's feces which had been fed to the assay rat in a 0.54 gm. daily dosage. Presumably

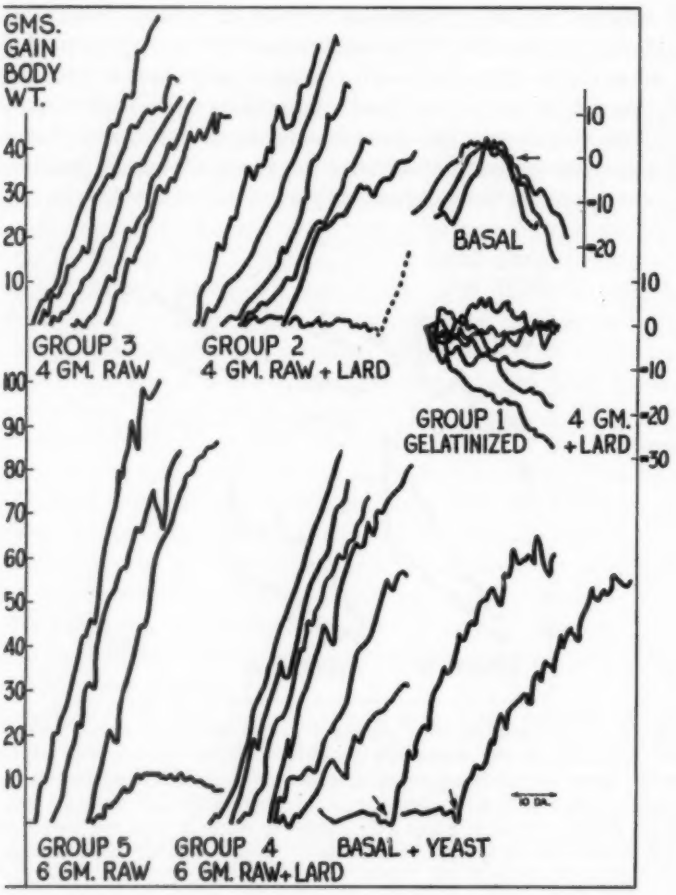

Fig. 2 Assays of the vitamin B content of daily collections of feces of donor rats shown in figure 1. The preliminary depletion period used for all assay rats is represented in this figure only for the groups in the negative and positive control experiments, respectively; for the others the curves begin with the feeding of the test doses. Note the strikingly greater elimination of vitamin B in the feces of the rats on raw potato starch in comparison with those on gelatinized, and the lack of effect of introducing 10% of lard into the ration. ←, marks the standard point of depletion of vitamin B in the negative and positive control experiments which was chosen for the beginning of dosage in the assays. ----- in the last curve in group 2 indicates a change in dosage, the feces of another donor rat being substituted for those which failed to promote growth.

this particular donor rat had not become 'refected' on raw starch as was evidently true also for one donor in group 5.

Some assay rats were fed feces in uniform daily amounts that had been produced on a 3.5 gm. level of the starch rations.

TABLE 1

Showing details of the assays for vitamin B in the feces and organs of rats on equalized intakes of potato starch rations

IDENTITY OF GROUPS AND RATIONS	INITIAL BODY WEIGHT OF DONOR RATS		TOTAL WEIGHTS DRY ORGANS FED IN 15-DAY PERIODS, THREE DONOR RATS TO EACH SET ORGANS	ASSAY RATS		AVERAGE WEIGHT DAILY COLLECTIONS FECES FED
	Average for total group contributing feces	Average for total group contributing organs		15-day gain in body weight in assay rats fed each lot organs	Average 30-day gain body weight assay rats fed feces	
1 Gelatinized starch + lard 4 gm. intake	gm. 66	gm. 74	gm. 3.5 4.7 3.4	gm. — 16 — 12 — 25	gm. — 10	gm. 0.16
2 Raw starch + lard 4 gm. intake	60	81	4.2 4.1	6 — 9	44	0.54
3 Raw starch low fat 4 gm. intake	66	84	3.7 4.0	12 17	56	0.49
4 Raw starch + lard 6 gm. intake	65	85	6.7 5.9	29 0	73	0.74
5 Raw starch low fat 6 gm. intake	63	86	5.7 5.6	29 3	70	0.89

The assay rats fed 0.5 gm. of feces from rats eating gelatinized potato starch showed practically the same rate of growth as the assay rats fed 0.5 gm. of feces produced on equal amounts of raw starch; these portions of feces collected on gelatinized starch represented as much as triple the length of time of the

feces collections on raw starch. Hence the elimination of vitamin B was three times greater on raw starch than on gelatinized.

The magnitude which is evident in the variations in the vitamin B content of the feces within any one of the groups of rats fed uniform amounts of a raw potato starch ration (fig. 2) tends to substantiate Fridericia's hypothesis of the nature of refection rather than that of Mendel and Vickery; an unequal consumption of 'residual' vitamin B is ruled out as a cause for the sharp difference between the averages in groups 1 and 2 and, on the other hand, the range in the fecal elimination of vitamin B in group 2 would be expected if it depends upon a synthesis in the intestine.

Inasmuch as a consumption of feces was not rendered entirely impossible in these experiments, it might be postulated that this had been practiced by group 1 of the donors to a greater extent than by group 2 and thus accounted for the difference in the apparent vitamin B-elimination between the two; this might seem the more probable because the average weight of the daily collections of feces for group 2 was about three and a third times that of group 1 (see table 1). However, if coprophagy had accounted for a disappearance of vitamin B from the feces collection of group 1, the vitamin B-content of the organs of this group would presumably have been correspondingly higher than in group 2. Table 1 shows this not to have been the case as the organs of group 2 were richer in vitamin B than those of group 1; therefore, coprophagy is ruled out as a factor of significance in the low content of vitamin B in the feces of the group fed gelatinized potato starch. It is not necessarily eliminated, however, as a source of some of the store of vitamin B in the organs of the rats on raw starch; the experiment therefore does not contribute evidence as to whether the refected rat obtains vitamin B through direct absorption of the vitamin at the site of synthesis, presumably in the coecum, or through a consumption of some of the vitamin rich fecal material.

The results in figure 2 and table 1 on the assay of the feces of groups 2 and 4 in contrast to groups 3 and 5 of the donor rats show unmistakably that the presence of lard in the ration had no measurable influence in increasing the total content of vitamin B in the rats' feces under the conditions of these experiments, hence the assumption of Whipple and Church that their observation on the concentration of vitamin B in the feces of their animals on rations varying in fat content explains the phenomenon of refection is without justification. Caution is necessary, however, in making interpretations from the present results in regard to other relationships of the fat of the diet to the metabolism and elimination of vitamin B inasmuch as certain variables were introduced by the nature of the study. It is worthy of comment, nevertheless, that in these few assays no tendency is apparent for the body organs studied to show a higher content of vitamin B when the restricted fat of the diet is increased by 10% of lard; the effect of synthesis in the intestine may well have masked such a tendency if it was present. However, the question might be raised as to whether or not the magnitude of the postulated effect of dietary lard in checking the losses of vitamin B from the body stores of rats in the experiments of Evans and Lepkovsky ('35) may not have been influenced, in some degree, by the effect on the intestinal flora of the much larger food intake of one of the fat-fed groups in contrast to the fat-low. This question arises particularly since these authors found that fecal elimination of the vitamin did not at all parallel the rate of its disappearance from the tissues. In the present experiments the effect of the amount of food intake is seen in that the rats on the 6-gm. intake tended to show a greater average fecal output of vitamin B than those on 4 gm. In view of the very wide variations within the individual groups, this difference, although occurring on rations which were admittedly not purified, seems to fit more logically with the assumption that it is attributable to a tendency for more food to reach the coecum than it does with the idea that the trace of preformed vitamin B introduced

into the ration by the 2% of rice polishings and possibly by the potato starch is significant. In the experiments of Evans and Lepkovsky one would be inclined to assume that the exclusion of starch from the ration ruled out all possibility of an influence of food intake on the degree of synthesis of the vitamin in the intestine and yet this is not entirely certain. The rations containing autoclaved acidified cornstarch in the experiments of Guerrant, Dutcher and Tomey ('35) appear to have supplied conditions for the synthesis of vitamin B in the digestive tract somewhat comparable to those in raw potato starch rations, although no explanation for this is at hand.

In some determinations of the vitamin B concentration in the feces of stock rats, very decided fluctuations were found from time to time although the composition of the ration remained uniform as far as was known; presumably these fluctuations were due to a varying synthesis of the vitamin rather than to a varying excretion. The uneven response obtained on a given stock ration at different times or in different laboratories may occasionally be due to a fluctuation in the synthesis of some factor rather than to variations in the foodstuffs or a gradual depletion of a factor in succeeding generations.

Results with starches other than potato. Paired feeding experiments (Seibert, '36) similar to those just described for potato starch but with corn and rice starches substituted in its place showed that no more vitamin B was eliminated in the feces of rats on these latter starches when the starch was fed raw than when it was gelatinized. This is in harmony with the fact that negative control experiments performed recently in this laboratory in vitamin B assays in which raw cornstarch was employed, have shown no variation from the results of former years, even though carried out in close proximity to refection experiments. The present studies give greater evidence for the etiological importance of the amount of unabsorbed food reaching the coecum of the rat than for the infectious nature of refection, postulated by others.

SUMMARY

1. The occurrence in rats on raw potato starch rations of a phenomenon with the characteristics of the 'refection' observed by Fridericia and others is described.

2. Gelatinization of the potato starch at temperatures not significantly destructive of vitamin B prevented the occurrence of refection.

3. On uniform intakes of a ration high in potato starch, rats tended to eliminate about three times as much total vitamin B in the feces when the starch was raw as when it was gelatinized.

4. The non-extractable fat of the starch is not a significant factor in the phenomenon of refection.

5. Some applications of these results to various problems in nutrition are suggested.

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A COMPARISON OF HEATED CASEIN WITH EXTRACTED CASEIN IN THE BASAL DIET FOR THE DETERMINATION OF VITAMIN A [1]

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The influence of heat on milk proteins has been widely studied. Greaves and Morgan ('34) showed that the lowered nutritive value of casein which had been heated was due to definite changes in the lysine and histidine fractions. Chick and associates ('35) reported that heating casein at 150°C. for 66 hours reduced the biological value from 64 to 45 when fed as 5% of the diet, and reduced the digestibility from 93 to 73%. Schultz, Seegers and Mattill ('35) observed that casein treated by heat or extraction does not permit optimum growth in experimental animals and they found that prolonged heating seemed to destroy or alter the nutritive value more completely than heating for a short period at a higher temperature. Fairbanks and Mitchell ('36) found the proteins of milk very sensitive to heat with respect to their value in nutrition; in the early stages of heating skim milk powder, cystine was destroyed but in the later stages when scorching occurred, the destruction of lysine exceeded that of cystine.

Casein extracted with boiling alcohol has been widely used as a source of protein in the vitamin A-free basal diet for rats. Giddings and Swim ('34) have pointed out the variability in the reaction of rats in vitamin A experiments and the lack of a standard method of extraction of the casein.

[1] Published as scientific paper no. 354, College of Agriculture and Experiment Station, State College of Washington.

In a study of a number of methods of extracting casein they showed that the Sherman and Smith ('31) method of extraction resulted in the removal of the maximum amount of vitamin A from the constituents of the basal diet.

Fraps ('31) in his vitamin A studies used casein which had been heated to 110°C. for 24 hours and reported that results were satisfactory when this casein was compared with that purified by extraction, but he presented no experimental evidence on this point.

Casein heated for 7 days at 110°C. has been recommended by Potter ('32) as a source of protein, free from vitamin A. Potter reported no differences in experimental findings when using heat-treated casein in place of alcohol-extracted casein. Her studies were not, however, made on litter mate controls and Coward, Key and Morgan ('33) have shown that variable responses may be obtained in animals of the same laboratory from year to year and even from litter to litter. It was therefore considered advisable to verify Potter's ('32) work, eliminating possible seasonal or hereditary differences and making direct comparison of the response of a group of animals receiving casein, heat-treated according to the method of Potter ('32), with the response of another group receiving casein, alcohol-extracted according to the method recommended by Giddings and Swim ('34). Conclusive evidence of the reliability of the use of heat-treated casein in vitamin A-free diets would be of value to those laboratories that lack the equipment for alcohol extraction of casein, and would also provide a cheaper method of preparation.

PROCEDURE

Albino rats of known nutritional history but from two different stock colonies were used in this study. Fifty-six rats were placed upon two experimental diets which were alike except for the treatment of the casein.

The vitamin A-free basal diet consisted of:

	%
Casein (freed from vitamin A)	18
Dried yeast[1]	10
Salt mixture (Osborne and Mendel)	4
Cornstarch	68
Each rat received 3 drops of viosterol per os weekly	

Diet I consisted of the above, using casein which had been heated for 7 days at 110°C. in a thermostatically controlled electric oven. The casein was heated in shallow pans, being stirred twice daily and systematically rotated to insure uniform heating throughout.

Diet II was similar to diet I except that the casein had been extracted with alcohol as follows: 1200 gm. of casein were placed in a 6-liter balloon flask with 3000 cc. of 95% alcohol and refluxed on a boiling water bath for 1 hour. An electrically driven stirring device was used to keep the casein agitated throughout the entire time. At the end of the hour the casein was filtered with suction, returned to the flask and 2800 cc. of boiling alcohol added while stirring. This was refluxed for 1 hour, filtered with suction, and again refluxed with 2800 cc. alcohol for 1 hour. The casein was filtered, washed on the filter with 3000 cc. boiling alcohol which was removed by suction and then spread out to dry under an electric fan at room temperature. Reclaimed alcohol was used for the first two extractions and new alcohol for the last extraction and washing.[2]

The rats were placed on the vitamin A-free diets at 21 to 28 days of age and weighing 35 to 50 gm. They were kept in individual cages throughout the experiment. Weight records of the animals were kept weekly for the first 3 weeks, after which time weighings were made every other day until the death of the animal. Distilled water was supplied at all times, the basal diet was always accessible, and records of food consumption were made each week. The time taken to

[1] From the Northwestern Yeast Company, Chicago, Illinois.

[2] We are indebted to Mr. Harvey Murer, research dairy chemist of the Washington Agricultural Experiment Station for preparing the extracted casein.

deplete the animals of their body stores of vitamin A was recorded in all cases and was determined by stationary or declining weight for 1 week and/or xerophthalmia. All animals were examined for external signs of vitamin A deficiency until death, and at autopsy the nose, middle ear, glands at the base of the tongue and neck, intestinal tract, kidneys, lungs and bladder were observed for macroscopic signs of infection.

The animals used were divided into three groups as follows:

Group A was composed of rats having a different hereditary and dietary background from the succeeding groups. Males only were available for use in this group, the females being used for fertility studies. The rats in this group were divided into two sub-groups and fed diets I or II; the average initial weight of rats on each diet was the same.

Group B was our regular stock colony animals, and both males and females were used. The rats in this group were evenly divided into two sub-groups of comparable weights and sexes and were fed diets I and II.

Group C was composed of a small number of rats of the same stock as group B. These animals were equally distributed between diets I and II and after depletion of their body stores of vitamin A they received in addition to the basal diet, 3 drops daily of cod liver oil. This quantity was considered sufficient to promote normal growth in the rat if all other dietary constituents were adequate. This supplementary feeding was continued for a period of 60 days at which time all animals were chloroformed and autopsied.

RESULTS AND DISCUSSION

In group A the depletion period averaged 28.6 days for the animals on diet I and 29.3 days on diet II with average weights of 98.5 gm. and 97.0 gm., respectively, at this stage. The total survival period, from the time the animals were placed on the vitamin A-free diets until death, together with the weight change during this period are here considered to be the best indications of the comparative value of the two

diets and therefore of the two types of casein. With diet I the average survival was 40 days with a final weight of 79.7 gm. and on diet II, 44 days and 73.7 gm.

For group B, the data for the males and females are recorded separately. The males were of greater initial weight than those in group A, they showed a longer depletion period of 44.9 days on diet I, and 44.3 days on diet II, and their average weights at depletion were greater, being 181.8 gm. and 179.3 gm., respectively. The total survival on the two casein diets was strictly comparable being 65.9 days and 65.0 days, with final weights at death of 131.7 gm. and 116.6 gm.

While the data secured with the two diets are in close agreement, the results from the two groups are different thus emphasizing the influence of hereditary and dietary background on the growth and survival of vitamin A-deficient rats. Such a difference also emphasizes the necessity of making simultaneous tests with a standard of reference and the unknown test food at all times in quantitative vitamin A determinations.

The females of group B showed similar weight responses, depletion time, and survival on the two diets, their weights, however, were lower, and the survival period longer than for the corresponding males.

The average food consumptions on the two diets in both groups are similar and therefore could not be considered an influencing factor.

The data for these groups are summarized in table 1.

Xerophthalmia, pus in the middle ear, in the nose and in the glands of the neck and base of the tongue are among the frequently occurring signs of vitamin A deficiency at the death of the experimental animals. A comparison of the occurrence of these abnormal conditions for all animals showed a somewhat higher incidence on diet II though the difference is probably not significant. On diet I for both groups the percentage incidence of infections was: ears 75.9, eyes 86.0, nose 75.9, tongue 62.0, and on diet II: ears 80.9, eyes 92.0, nose 84.3 and tongue 69.2.

TABLE 1

Comparison of growth, survival and food consumption of rats on diets containing (I) heated and (II) alcohol-extracted casein

DIET	ANIMALS		BODY WEIGHT					DEPLETION PERIOD, AVERAGE	TOTAL SURVIVAL		FOOD CONSUMPTION PER DAY
	Description	Number used	Initial		Depletion average	Final			Average	Range	
			Average	Range		Average	Range				
			gm.	*gm.*	*days*	*gm.*	*gm.*	*days*	*gm.*	*days*	*gm.*
I	♂, group A	11	40.5	33–49	98.5	79.7	54–110	28.6	40.0	26–50	7.6
II	♂, group A	11	42.0	33–46	97.0	73.7	64–100	29.3	44.0	33–61	7.3
I	♂, group B	8	45.0	40–52	181.8	131.7	102–150	44.9	65.9	52–87	10.3
II	♂, group B	7	45.3	40–51	179.3	116.6	101–140	44.3	65.0	57–72	10.4
I	♀, group B	9	44.4	37–52	148.5	97.1	72–118	45.1	76.6	64–93	9.3
II	♀, group B	10	42.9	34–51	152.1	98.6	81–118	44.8	76.8	57–98	9.8

From this evidence it is clear that heat-treated casein may be substituted for alcohol-extracted casein in the vitamin A-free basal diet when judged by the growth response, depletion period and total survival time of the experimental animals serving as negative controls.

The animals in group C were used to determine whether the heat-treatment of the casein had changed the nature of the protein or had caused sufficient destruction of essential amino acids to prevent normal growth responses when vitamin A was added as a supplement to the basal diet. If such were

TABLE 2

Comparison of growth and food consumption of vitamin A-depleted rats receiving diets I and II supplemented with cod liver oil

DIETS	RAT NO.	EXPERIMENTAL PERIOD	TOTAL WEIGHT GAIN	WEEKLY WEIGHT GAIN	FOOD PER DAY	WEIGHT GAIN PER GRAMS FOOD
		days	*gm.*	*gm.*	*gm.*	*gm.*
I + 3 drops cod liver oil daily	♂ 520	60	232	27.1	18.6	0.208
I + 3 drops cod liver oil daily	♂ 525	60	148	17.3	15.6	0.158
I + 3 drops cod liver oil daily	♀ 523	60	83	9.7	15.7	0.088
I + 3 drops cod liver oil daily	♀ 524	60	67	7.8	15.7	0.071
I + 3 drops cod liver oil daily	♀ 526	60	77	9.1	12.3	0.104
Average for three females			75.7	8.8	14.5	0.087
II + 3 drops cod liver oil daily	♀ 521	60	85	9.9	15.1	0.094
II + 3 drops cod liver oil daily	♀ 522	60	70	8.1	13.3	0.088
II + 3 drops cod liver oil daily	♀ 527	60	73	8.5	14.8	0.082
Average for three females			76.0	8.9	14.4	0.088

the case the use of heat-treated casein might result in misinterpretation of growth responses when making experimental determinations on the vitamin A values of unknown foods or test substances.

The results obtained from group C animals are summarized in table 2. The number of animals used was small but the close agreement of the data from the two diets is believed to justify the conclusion that there was no difference in the two diets. The two males made weight gains which are comparable to those of normally growing rats and the females in both series made average weekly gains of 8.8 gm. and the food consumption was the same in both cases.

SUMMARY

A comparison was made of alcohol-extracted casein and heat-treated casein as a source of protein in the vitamin A-free basal diet of carefully matched experimental rats.

No marked differences were observed in the growth responses, time for depletion or survival period of groups of animals on the two diets, with and without added vitamin A supplement.

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ALLEVIATION OF VITAMIN B DEFICIENCY IN THE RAT BY CERTAIN NATURAL FATS AND SYNTHETIC ESTERS

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TWO FIGURES

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Whether fats affect the requirements of animals for vitamin B is very important from the viewpoint of the worker who is investigating the physiological function of this vitamin. That fats decrease the requirement for vitamin B was first reported by Evans and Lepkovsky ('28, '29). Data in support of their initial findings have been submitted by several investigators (Evans and Lepkovsky, '31, '32 a, b, c, d, e, f, '35; Evans, Lepkovsky and Murphy, '34 a, b; Salmon and Guerrant, '30; Salmon and Goodman, '31; Guerrant and Dutcher, '34; Whipple and Church, '36).

Other investigators, however, have reported conflicting or contradictory results (Gregory and Drummond, '32; Kemmerer and Steenbock, '33; Westenbrink, '34 a, b, '35; Sure and Buchanan, '35).

The presentation of further data relating to the problem would, therefore, seem to be pertinent. It is the purpose of this paper to report the most significant of such data which have been obtained in this laboratory and to discuss them in relation to the results obtained in other laboratories.

EXPERIMENTAL MATERIALS AND METHODS

Natural fats and oils. The coconut oil and the hydrogenated cottonseed oil were edible grades obtained from the Capital City Products Co., Columbus, Ohio. Olive oil and cottonseed

oil (Wesson oil) were purchased on the local market. Butter was obtained from the A. P. I. Creamery; it was melted and filtered free from curd and water. The two samples of lard were obtained from the Bureau of Animal Industry Laboratory at Beltsville, Md. The pecan oil was prepared in our laboratory by extracting ground pecan kernels with high test (gas machine) gasoline, removing the solvent, and filtering the oil. Beef fat was likewise prepared by rendering beef suet in an open kettle and filtering.

Prepared esters. The ethyl esters, and glyceryl acetate, propionate, and butyrate were purchased from Eastman. The other glycerides were prepared in our laboratory. The lactic acid was Baker's C. P.; the other acids were the highest grade obtainable from Eastman. The glycerol was Merck's reagent, anhydrous.

A 20% excess of glycerol was used in the esterifications. The temperature varied slightly because of differences in the boiling points of the acids. For the short chain acids the reaction was carried out at atmospheric pressure at a temperature just under the boiling points of the acids. For the longer chain acids the temperature ranged from 170°C., where the reaction usually began, to 240°C. with about a 5-pound reduction in pressure. Agitation of the reaction mixture was accomplished by a stream of dried CO_2 which facilitated removal of the water and prevented oxidation.

When the reaction became impracticably slow the heating was discontinued as it was considered desirable to avoid prolonged heating. The time varied from 5 to 7 hours after the reaction started. After the mixture cooled to 100°C. in an atmosphere of CO_2, 2 gm. of norite and 5 gm. of fuller's earth per 500 gm. original acid were added and after thorough agitation the mixture was filtered. The filtrate was washed with three changes of hot water and again filtered. The acid number was then determined. The lauric, myristic, palmitic and stearic glycerides were dissolved in twice their volume of hot alcohol; the mixture was allowed to cool and as much of the alcohol as possible was drawn off. The alcohol treatment was repeated three times. After the last treatment, the

remaining alcohol was washed from the fat with hot water. The shorter chain esters received an addition of KOH sufficient to neutralize the free acid, the strength of the alkali depending upon the free fatty acid content (Jamieson, '32). The mixture stood at room temperature until the soap separated, after which the soap was removed and the fat was washed three times with water and filtered. The free acid

TABLE 1

Source of acid, saponification number and percentage of acid in esters

ESTER	SOURCE OF ACID [1]	SAPONIFICATION NUMBER	PER CENT ACID
G. acetate	.....	771.0	82.5
G. propionate	.....	646.0	85.3
G. butyrate	.....	557.0	86.8
G. valerate	Butyl cyanide	462.7	84.1
G. caproate	Amyl cyanide	418.9	86.7
Et. caproate	Amyl cyanide	395.0	81.7
G. iso-caproate	.....	412.2	85.2
G. heptoate	Heptyl alcohol	372.5	86.4
G. caprylate	Coconut oil	351.1	90.2
G. nonylate	Heptyl bromide and ethyl malonate	317.5	89.5
G. undecylate	Undecylenic acid	281.8	93.5
G. laurate	Coconut oil	263.4	94.0
G. myristate	Nutmeg wax	226.5	92.0
G. palmitate	Animal fat	212.3	96.9
Et. palmitate	Animal fat	201.1	91.8
G. stearate	Animal fat	194.0	98.3
Et. stearate	Animal fat	196.9	94.7
G. oleate	.....	187.0	94.1
Et. oleate	.....	185.4	93.3
G. lactate	.....	469.7	75.2
Coconut oil	.....	260.5	93.0

[1] From information furnished by Eastman Kodak Co.

was not neutralized in the glyceryl oleate or lactate nor was the excess glycerol washed out of the lactate. The liquid glycerides were neutral after the refining was completed; the lauric, myristic, palmitic and stearic glycerides contained from 3 to 7% of free acid. The saponification number of all preparations was determined as a basis for calculating the amount of ester to be fed. Table 1 shows the saponification value

and the percentage of acid in the various esters which were used in this study.

Other dietary ingredients. Number 1 casein from Adler and Son, Philadelphia, was used after purification (Salmon and Goodman, '34). The sucrose was commercial refined cane sugar.

The autoclaved yeast was Northwestern powdered yeast.[1] Before being autoclaved it assayed 3–3.3 international units of vitamin B per gram by the pigeon protective method (Salmon, '27). The dry yeast was extracted with gasoline until no more color was removed. The residual gasoline was allowed to evaporate from the yeast which was then spread in layers about 12 mm. deep in enameled pans; the yeast was moistened, autoclaved 8 hours at 120°C., dried at 70°C., ground and stored in tightly closed tinned cans until used.

The brewer's yeast was Vitamin Food Co., no. 1 debittered yeast.[1] It assayed 30 international units of vitamin B per gram by the above method. The vitamin B solid was prepared from an extract of the brewer's yeast by adsorption on fuller's earth at about pH 3.5. It assayed 300 international units per gram.

The diets. Table 2 shows the composition of the diets. In general all the ingredients of the diets were mixed together except the autoclaved yeast, the cod liver oil and the linseed oil; in the diet 55% series, however, the oils and liquid esters were pipetted into the feed jars daily onto weighed portions of the dry mixture of other ingredients. This was necessary to avoid changes in the composition of the diet by settling. The cod liver oil was mixed into diet 40 fat; but in all the other diets both the cod liver oil and the linseed oil were pipetted directly into the feed jars daily. The autoclaved yeast was fed daily in separate jars.

The animals. The rats (Wisconsin strain) were started on test at 23 to 26 days of age at as near 55 gm. average weight

[1] We wish to express our appreciation to Dr. M. H. Givens of the Northwestern Yeast Company and to President R. M. Allen of the Vitamin Food Company for the yeast used in these studies.

as possible. They were kept in individual cages with raised screen floors, 3 meshes to inch. Feed and distilled water were supplied daily. Weights of rats were recorded weekly.

Digestibility of fat. After the rats had received the fat diets for 1 week, the feces were collected for 4 weeks, except where death of the rats necessitated a shorter collection period. The feces from all of the rats on a given diet were pooled and dried at 45°C. They were then weighed and aliquots (or all the feces from a group if the amount permitted) placed in Soxhlets. They were extracted with ether for 24 hours, then

TABLE 2

Percentage composition of basal diets

INGREDIENTS	DIET						
	Low fat	High fat	40 fat	5%[2]	23%[2]	36%[2]	55%[2]
Purified casein	18.0	32.0	22.5	18.0	18.0	22.5	32.0[3]
Salt 186[1]	4.0	7.0	5.7	4.0	4.0	5.7	7.0
Agar	1.0	1.8	1.0	1.0	1.0	1.0	1.8
Fat or ester	....	59.2	40.0	5.0[4]	23.0[4]	36.0[4]	55.0[4]
Sucrose	77.0	....	28.8	....[5]	....[5]	....[5]	

[1] J. Biol. Chem., 1930, vol. 89, p. 199.

[2] Indicates fatty acid equivalent of ester or fat in diet.

[3] In the case of glyceryl acetate it was necessary to reduce the casein to 28, salt to 5, agar to 1% in order to get the equivalent of 55% acetic acid in diet.

[4] These figures represent the fatty acid equivalent of the esters; the percentage of ester was necessarily affected by the molecular weight of the fatty acid.

[5] Sucrose was added to make 100%.

made acid to Congo red with a few drops of concentrated H_2SO_4 and again extracted with ether for 12 hours. After the ether was dissipated the two fractions of extract were weighed. From these weights and the amount of fat ingested during the period the percentage of fat retained was calculated.

Preparation and examination of tissue fats. The fatty acids from livers and brains of several groups of rats were used for magneto-optic (Allison and Murphy, '30) and refractive index determinations. These rats were killed by decapitation; the livers and brains were removed as rapidly as possible and dissolved in 10 ml. of boiling 60% KOH. The solutions were

heated in covered flasks for 4 hours on a water bath. When cool, each sample was made to 100 ml. and 1 ml. removed and diluted for magneto-optic study. The remainder was acidified with HCl and shaken with three 25-ml. portions of ether. The ethereal solutions were washed with water and the three fractions combined. After the ether had dissipated, the dry material was extracted with three 10-ml. portions of ether which were filtered through dry fat-free filter paper. The filtrates were evaporated to dryness. The residue, designated as the fatty acid fraction, was weighed and the refractive index was determined at 60°C. with an Abbé refractometer.

RESULTS

Effect of coconut fat. The approach to the problem under consideration was to determine whether the addition of various fats to a vitamin B-free diet would increase the gain in weight or the time required for rats to develop beriberi. Beriberi is used throughout this paper for the onset of symptoms which appear in the late stages of vitamin B deficiency. It is characterized by varying degrees of anorexia, marked incoordination or muscular weakness, and finally by prostration and death within a few days unless treatment is given. Spasticity almost invariably appears in the animals which develop beriberi on diets containing moderate amounts of fat; it sometimes is not observed in animals receiving a vitamin B deficient diet which is low in fat, in which cases the picture may be one of inanition and general weakness.

The weight curves in figure 1 show that the diet containing 23% of coconut oil produced a slightly larger initial gain than the low fat diet but did not delay the onset of beriberi. A diet containing 59.2% of the oil, however, not only increased the gain significantly but had a pronounced effect in inhibiting the symptoms of beriberi. Of the six rats started on this diet, only one developed beriberi and that was after 26 weeks. This was more than four times as long as was required by the rats receiving the low fat diet or the diet containing only 23% of coconut fat. The experiment was continued for 48 weeks

without another case of beriberi developing. These rats received only 250 mg. of autoclaved yeast per rat daily which was the same amount received by those on the low fat and the 23% fat diets. Three other groups of rats were fed on the 59.2% fat diet to determine the effect of increasing the autoclaved yeast to 500 mg. daily as well as the effect of adding vitamin B. The weight curves for these are also shown in

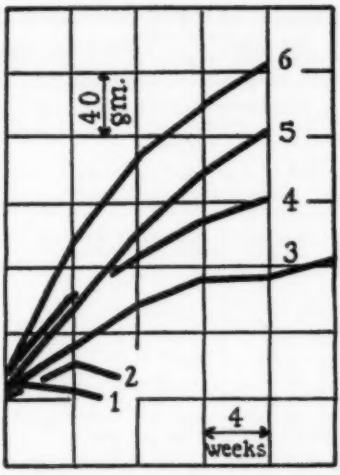

Fig. 1 Composite growth curves of rats. Curve 1, low fat diet with 250 mg. autoclaved yeast; curve 2, 23% coconut oil with 250 mg. autoclaved yeast; curve 3, high fat (59.2% coconut oil) with 250 mg. autoclaved yeast; curve 4, high fat with 500 mg. autoclaved yeast; curve 5, high fat with 250 mg. autoclaved yeast and 10 mg. vitamin B solid; curve 6, high fat with 500 mg. autoclaved yeast and 10 mg. vitamin B solid per rat daily. The ends of curves 1 and 2 indicate death of the last rat in group. The rats represented by curve 3 were continued for a total of 48 weeks with but one case of beriberi. Those represented by curves 4, 5 and 6 were discontinued after 16 weeks. Curve 6 represents two rats; other curves, six rats each.

figure 1. Although increasing the autoclaved yeast from 250 mg. to 500 mg. per day improved the rate of growth, the improvement was less than that resulting from the addition of 10 mg. of vitamin B solid to the 250 mg. of autoclaved yeast. Moreover, there was a further stimulation of the growth rate

by adding 10 mg. of vitamin B solid to the 500 mg. level of autoclaved yeast.

It is thus apparent that the high fat diet is improved by the addition of vitamin B or some other heat labile factor carried by the solid. It also appears that at the 250-mg. level of autoclaved yeast the limitation of growth is more dependent upon a lack of this factor than of vitamin G.

Effect of different levels of autoclaved yeast. That increasing the level of autoclaved yeast increased the growth produced by the high fat diet was shown in figure 1. Further data in table 3 show this more strikingly. The effect of the increased level of autoclaved yeast was much greater with the

TABLE 3

Average gain in weight and time to onset of beriberi in rats receiving different levels of autoclaved yeast[1]

DIET	YEAST DAILY	NUMBER OF RATS	AVERAGE TOTAL GAIN	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET	LENGTH OF TEST
	mg.		*gm.*	*%*	*days*	*days*
High fat[2]	250	4	61	25	107	127
High fat[2]	500	4	128	0	...	127
High fat[2]	1000	4	164	0	...	127
Low fat	250	6	2	100	37	45
Low fat	500	6	6	83	59	119

[1] Each rat received 0.10 ml. cod liver oil per day.

[2] Coconut oil was used in the high fat diet in these experiments.

high fat diet than with the low fat diet. Even with the latter there was a considerable delay in the onset of beriberi. Our interpretation of these results is that the autoclaved yeast still contained traces of vitamin B. Since the requirement of the rat for vitamin B is small, the effects of such traces become apparent when large amounts of autoclaved yeast are fed. The effects are emphasized by the peculiar action of fat.

Comparison of different fats. A series of preliminary experiments was conducted to determine the best level of fat for comparing the effects of fats when added to a vitamin B free diet. On the basis of these tests a level of 40% of the test fat in the diet was selected. Table 4 includes the results of this comparison.

Coconut oil was the most effective of the natural fats both in producing gain in weight and in delaying the onset of beriberi. Cottonseed oil and pecan oil ranked next in delaying the development of beriberi but did not produce as much growth as lard or butter. The latter ranked at the bottom of the list in its ability to delay the occurrence of beriberi but was next to coconut oil in its effect on growth. It is probable that palatability of the fat may affect growth more than it does the

TABLE 4[1]

Average gain in weight and time to onset of beriberi in rats receiving vitamin B deficient diet (40 fat) containing various fats at a level of 40%[2]

KIND OF FAT	NUMBER OF RATS	AVERAGE TOTAL GAIN	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET
		gm.	*%*	*days*
Myristin 30 Hard lard 10	6	47	0	..[3]
Coconut	6	28	83	60
Cottonseed	11	6	100	51
Pecan	6	9	100	46
Hyd. cottonseed	24	12	95	40
Linseed	8	5	100	40
Hard lard	18	20	83	40
Soft lard	6	17	100	37
Hard lard 30 Myristin 10	6	17	100	37
Beef	11	5	100	35
Olive	6	14	100	35
Butter	7	21	100	34

[1] The data in this table were taken from a master's thesis submitted to this institution by J. G. Goodman.

[2] Each rat received 250 mg. autoclaved yeast and 0.10 ml. cod liver oil per day.

[3] No beriberi at end of 102 days.

time required for the onset beriberi. The differences in the natural fats with the exception of coconut and cottonseed oil do not seem to be important. The effect of a mixture of 3 parts of myristin and 1 part of lard, however, is very striking.

Comparison of various esters. A series of tests on synthetic esters of various fatty acids was next undertaken. Most of these esters were fed at a level equivalent to 23% of fatty acid in the diet. Glyceryl caprylate was also fed at the equivalent of 5, 36 and 55% and glyceryl caproate at the equivalent

of 5% fatty acid. The essential unsaturated fatty acids were supplied by 0.20 ml. of U. S. P. linseed oil per rat daily. All rats in this series were subjected to a preliminary, 2-week depletion period on the low fat diet in an attempt to reduce their body stores of vitamin B to a low level before the feeding of the ester-containing diets was begun. It was expected that all rats receiving diets containing only 23% of the esters would succumb to the deficiency and that the time of onset of premortal symptoms as well as the amount of growth could be used in evaluating the esters. As the experiment progressed it became apparent that some of the esters might prolong life indefinitely. Consequently the feeding of the esters was discontinued after 12 weeks in all groups that had not developed beriberi previous to that time.

Table 5 presents the results of this series. At the 23% level glyceryl caprylate produced significantly more growth than any of the other esters. Glyceryl caproate ranked next, the iso-caproate apparently being as good as the normal. Ethyl caproate produced only slightly less growth than the glyceryl ester. It is interesting that glyceryl heptoate produced nearly as much growth as the caproate; the heptoate gave about the same growth as the laurate and slightly better than the myristate which did not produce significantly more gain in weight than the nonylate and the undecylate. The only cases of beriberi which occurred at the 23% level were on ethyl palmitate, glyceryl palmitate, stearate, and acetate, and coconut fat which was included in the series as a control. Of eighteen rats on the low fat diet as controls, fourteen developed beriberi in an average of 43 days. As compared with this the palmitate, stearate, and acetate actually decreased the time required for the onset of beriberi. The acetate stimulated growth slightly but the palmitate and stearate produced digestive disturbances and a steady loss of weight. The butyrate also decreased the survival period but the symptoms suggested a toxic effect rather than vitamin B deficiency. Later tests showed a similar toxic action in the presence of an adequate vitamin B intake.

TABLE 5

Average gain in weight and time to onset of beriberi in rats receiving vitamin B deficient diets[1] containing various glyceryl and ethyl esters

DIET	ACID EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN 6 WEEKS[4]	AVERAGE GAIN 12 WEEKS[4]	BERIBERI INCIDENCE	AVERAGE TIME TO ONSET[4]	ESTER RETAINED
Series A[2]	%		*gm.*	*gm.*	%	*days*	%
G. caprylate	55	4	57	Discontinued	0	..	99.2
G. caprylate	36	4	51	Discontinued	0	..	....
G. caprylate	23	4	57	101	0	..	98.4
G. caprylate	5	4	47	75	0	..	....
G. caproate	23	4	46	72	0	..	99.0
G. iso-caproate	23	4	46	Discontinued	0	..	98.7
Et. caproate	23	4	42	64	0	..	....
G. heptoate	23	4	38	64	0	..	98.0
G. laurate	23	4	31	63	0	..	97.4
G. myristate	23	4	28	47	0	..	96.3
G. nonylate	23	4	23	42	0	..	98.4
G. undecylate	23	4	20	40	0	..	98.8
Et. oleate	23	4	23	34	0	..	96.9
G. propionate	23	4	19	33	0	..[5]	98.9
G. lactate	23	4	17	27	0	..	94.6
G. oleate	23	4	15	25	0	..	97.4
G. caproate	5	4	12	32	0	..	....
G. valerate	23	4	17	17	0	..[6]	98.7
Low fat diet	..	18	7[7]	Died	77	43	93.5
Coconut	23	8	13[7]	Died	62	40	97.6
Et. palmitate	23	4	0[8]	Died	75	38	92.6
G. acetate	23	4	24[7]	Died	100	32	99.0
G. palmitate	23	4	0[8]	Died	100	30	91.5
G. stearate	23	4	0[8]	Died	100	17	74.4
G. butyrate	23	4	0[8]	Died	...	20[9]	97.6
Series B[3]							
G. caprylate	5	4	10	11	25	63	....
Low fat diet	..	14	0[8]	Died	100	25	....

[1] Each rat received 0.10 ml. cod liver oil and 0.20 linseed oil per day.

[2] Each rat in series A received 500 mg. autoclaved yeast per day.

[3] Each rat in series B received 250 mg. autoclaved yeast per day.

[4] All rats received the low fat diet for a 2-week depletion period; the time of experiment was reckoned from the end of this period.

[5] One rat died 3 days after starting on glyceryl propionate.

[6] One rat died 3 days after starting on glyceryl valerate.

[7] Average gain to maximum weight.

[8] These rats lost weight from start of ester-containing diets.

[9] Average length of life; symptoms not typical of beriberi.

Glyceryl caprylate was also fed at levels corresponding to 5, 36 and 55% of caprylic acid in the diet. The growth at 6 weeks was no better on the higher percentages than on the 23% level. On 5% of glyceryl caprylate the growth was about 17% less at 6 weeks and 25% less at 12 weeks than on 23%. As at the 23% level, glyceryl caproate at the 5% level was less effective in supporting growth than the caprylate.

When the dosage of autoclaved yeast was reduced to 250 mg. per rat daily, glyceryl caprylate at the 5% level did not entirely prevent beriberi, although only one rat in a group of four went down in the 12-week test period. In contrast with this, fourteen rats on the low fat diet and likewise receiving 250 mg. of autoclaved yeast showed a 100% incidence of beriberi; the average time required was 25 days after the beginning of the test period.

In view of the belief of some investigators that vitamin B may function in the metabolism of lactic acid, it is interesting to note the effect of this acid. None of the rats receiving glyceryl lactate equivalent to 23% of lactic acid in the diet, developed beriberi in the 12-week test period. Either less vitamin B was required in the metabolism of lactic acid than of sucrose or the c. p. lactic acid carried traces of vitamin B as an impurity.

The percentage of food fat retained was high and apparently too uniform to account for the differences in efficiency of the esters. The only marked reductions in the retention were with the esters of palmitic and stearic acids. There were always evidences of digestive disturbances when the esters of these acids were fed. It is possible that unabsorbed residues of these substances which melt above the body temperatures are irritating to the intestinal mucosa. The apparent poor retention of food fat with the low fat diet is probably due to the excretion of fatty material through the intestinal tract. This would obviously cause a greater error with a low fat diet than with a moderate or high fat diet.

Curative tests with glyceryl caprylate and caproate. Since some of the esters in the preceding tests were so potent in

preventing beriberi, it was decided to determine what would be the effect of feeding them to rats which had developed the disease on other diets.

The curative action of diets containing 23% of glyceryl caproate or caprylate was very striking. The rats whose weight curves are shown in figure 2 were in the spastic stage

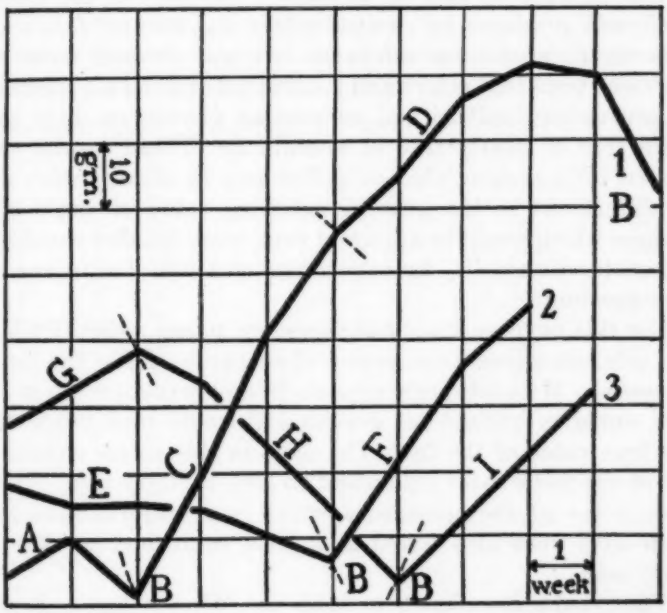

Fig. 2 Composite growth curves of rats. Curve 1: A, 23% glyceryl acetate; C, 23% glyceryl caprylate; D, 23% glyceryl acetate. Curve 2: E, low fat; F, 23% glyceryl caproate. Curve 3: G, 23% glyceryl iso-caproate; H, low fat; I, 23% glyceryl iso-caproate. B indicates onset of beriberi. Curve 1 represents one rat; curve 2, two rats; curve 3, three rats.

of beriberi. It was necessary to turn the feed jars on the side and support the rats for their first feeding of the ester-containing diets. The cures were nearly as rapid as when brewer's yeast was fed. These curative tests apparently show that to a certain extent there may be an actual substitution of fatty acids for vitamin B and carbohydrate in the

metabolism of the rat. One curve in figure 2 is particularly interesting: it shows that when 23% of acetic acid as the glyceride was fed, beriberi developed and was cured by substituting caprylic for acetic ester but again developed when the feeding of the acetic ester was resumed. Numerous other cures were effected, the results indicating a correlation between the curative and the protective efficiency of the esters.

Growth produced by various esters and natural fats when vitamin B intake was adequate. It was evident from the previous tests that esters and natural fats varied significantly in preventing beriberi and supporting growth on diets that were free or nearly free of vitamin B. These results suggested the question whether differences in efficacy were due to differences in the general nutritive value of these substances which would be apparent even when the diet contained adequate vitamin B. An experiment was initiated to answer this question.

For this purpose it seemed necessary to use a diet in which the sole non-nitrogenous source of energy would be the fat to be tested. With adequate vitamin B, any carbohydrate in the diet would be utilized for growth and would tend to obscure the true value of the fat. The diets in this series contained fat or synthetic ester equivalent to 55% of fatty acid. However, some of the substances which were injurious at this high level were also tested at a level equivalent to 23% of fatty acid.

The results are shown in table 6. When vitamin B was supplied, coconut oil, olive oil and lard were of practically identical values as sources of energy. Moreover none of these were significantly superior to sucrose, at least within the 6-week test period. Beef fat, cottonseed oil and butter fat were slightly less valuable than the above. Glyceryl caproate or laurate were distinctly inferior to any of the natural fats or to sucrose.

A mixture of equal parts of glyceryl heptoate and nonylate gave poor results, two of the rats dying before the end of the test period. Two rats died on the glyceryl acetate diet also.

Glyceryl palmitate was even more harmful; the average life of the four rats on this diet was only 8 days.

Glyceryl acetate equivalent to 23% acetic acid lowered the nutritive value of the diet markedly but less than glyceryl or ethyl stearate at this level. Glyceryl butyrate was distinctly harmful, only one rat of four living 6 weeks.

TABLE 6

Average gain in weight and average food of rats receiving adequate vitamin B and diets containing various esters or natural fats[1]

ESTER OR FAT	ACID EQUIVALENT	NUMBER OF RATS	AVERAGE GAIN 6 WEEKS	AVERAGE FOOD 6 WEEKS	ESTER RETAINED
	%		gm.	gm.	%
Coconut	55	4	130	248	98.8
Olive	55	4	128	248	97.7
Lard	55	4	126	274	96.1
Low fat	55	4	124	391	89.8
Beef	55	4	114	253	94.9
Cottonseed	55	4	107	220	96.6
Butter	55	4	105	226	96.9
G. caproate	55	4	84	224	99.4
G. laurate	55	4	67	170	97.9
G. heptylate nonylate	55	4	65[2]	176	99.2
G. acetate	55	5	45[3]	216	87.2
G. palmitate	55	4	..[3]	...	....
G. stearate	23	4	54	295	19.1
Et. stearate	23	4	37	251	48.9
G. butyrate	23	4	—22[4]	...	98.2
G. acetate	23	4	90	349	99.3

[1] Each rat received 500 mg. brewer's yeast, 0.10 ml. cod liver oil, and 0.20 ml. linseed oil per day.

[2] Two rats died before end of 6-week period.

[3] Average life was 8 days on this diet.

[4] Only one rat lived 6 weeks on this diet.

These results show that fats do not rank the same when the diet contains adequate vitamin B as when it is deficient in vitamin B. Differences in their effects on rats receiving vitamin B deficient diets are not due to differences in their general nutritive value.

Effect of vitamin B deficiency on fats of brains and livers. We next tried to determine whether vitamin B or fat in the diet affects the nature of the fatty acids in the tissues of rats. Several groups of rats received controlled amounts of food and after suitable intervals their brains and livers were prepared for study as described under methods. Weights and refractive indexes of the fatty acid fraction from these tissues were determined and are shown in table 7.

TABLE 7

Average weights and refractive indexes (at 60°C.) of fatty acid fraction from brains and livers[1]

GROUP	NUMBER OF RATS	DIET	VITAMIN B[2]	AVERAGE WEIGHT OF RAT	BRAIN FATTY ACID		LIVER FATTY ACID	
					Average weight	Ref. ind.	Average weight	Ref. ind.
				gm.	*mg.*		*mg.*	
1	3	Low fat	—	73	84	1.4714	96	1.4644
2	3	Low fat	+	100	87	1.4680	173	1.4569
3	3	High fat[3]	—	80	47	1.4713	70	1.4670
4	3	High fat[3]	+	100	75	1.4709	213	1.4606
5	3	Low fat	—	43	88	1.4703	63	1.4618
6	2	Low fat	+	43	57	1.4750	54	1.4624
7	5	High fat[3]	—	48	60	1.4707	70	1.4629
8	3	High fat[3]	+	47	65	1.4718	70	1.4652
9	3	Low fat	+	198	101	1.4705	246	1.4535
10	4	Stock	+	64	57	1.4689	71	1.4626

[1] Each rat in groups 1 to 8, inclusive, received 0.10 ml. cod liver oil, 0.20 ml. linseed oil, and 250 mg. autoclaved yeast per day.

[2] Each rat in groups 2, 4, 6, 8 received 20 mg. vitamin B solid on alternate days; each rat in group 9 received 500 mg. brewer's yeast per day and the usual dosage of linseed and cod liver oils.

[3] Wesson oil was used in the high fat diet in this series.

The rats in groups 1 to 4, inclusive, were started on experiment simultaneously. All rats received vitamin B for 4 weeks. Groups 2 and 4 were then killed. The vitamin B was omitted and groups 1 and 3 were continued on their respective diets for 6 weeks. At this time all the rats in group 1 were losing weight and one rat was showing spastic symptoms of beriberi. The rats in group 3 were likewise losing weight due to limitations of their food since the energy intake was kept uniform for all four groups in this experiment.

The weights of the fatty acid fractions of both brains and livers were less in groups 1 and 3 which did not receive vitamin B than in groups 2 and 4 which received this vitamin. The most surprising feature, however, is the smaller weight of the fatty acid fraction of these tissues in group 3 which received the high fat diet without vitamin B than in group 1 which received the low fat diet without vitamin B. The refractive indexes were somewhat higher in the groups which did not receive vitamin B, the fat from the livers showing more difference than the fat from the brains.

The energy intake in groups 5, 6, 7 and 8 was likewise kept uniform but it was more rigidly limited than in the preceding groups. The maximum daily intake was kept at 12 Cal. per rat. As the deficient rats reduced their consumption below this amount, the diet of the rats receiving vitamin B was correspondingly reduced. It was found impossible to reduce the energy intake of the rats that received vitamin B to the low levels reached by the deficient rats in the incipient stages of beriberi; if this was done the rats receiving vitamin B always died before the ones not receiving vitamin B. Hence, 10.8 Cal. per rat daily was adopted as the minimum level for those which received vitamin B. It is interesting to note that group 6 receiving the low fat diet and vitamin B actually had less fatty acid fraction in both brains and livers than group 5 which received the same diet without vitamin B. The refractive indexes were also slightly higher for the group which received the vitamin. These relationships were reversed from those in groups 1 and 2 where the energy allowance was more liberal. The weights of the fatty acid fraction in groups 7 and 8 were not so divergent but the refractive indexes showed the same reverse relationship.

Group 9 received the low fat diet, ad libitum, and vitamin B for 10 weeks. Group 10 was a group of rats from the stock colony; they were just a few days older than the rats in the other groups were when placed on experiment. The refractive indexes in these two groups were not markedly different from those of the other groups, except they were quite low in the fatty acid fraction from the livers in group 9.

On the whole it does not appear that a deficiency of vitamin B had any direct influence on the refractive index of the fatty acid fraction from the brains or livers.

Data from the magneto-optic studies will not be presented in this paper. In groups 1, 2, 3 and 4 there was a lower content of caproic and caprylic acids in the brains and livers of the deficient rats than of those receiving vitamin B. In groups 5, 6, 7 and 8 this was reversed. In all cases the content of these acids was very low and the differences did not appear to be significant.

The magneto-optic minima which have been reported for formaldehyde (Sommer, '33) and formic acid (McGhee and Laurenz, '33) were observed in preparations from the brain, liver and blood of rats in all groups. The circle readings indicated a higher concentration of these compounds in the tissues of rats receiving the low fat diet than in those receiving the high fat diet; they also indicated a higher concentration of formic acid in the tissues of rats receiving vitamin B than in those of rats not receiving this factor. Although the existence of formic acid in animal tissues has been reported, we do not believe the presence of formaldehyde has been previously observed. If its presence can be confirmed by other methods, some very interesting possibilities regarding its role in intermediary metabolism will need investigation.

DISCUSSION

In comparing our results with those of other investigators it is well to keep in mind the possible influence of certain differences.

In the experiments of Evans and Lepkovsky and in our early experiments with natural fats no provision was made for a supply of essential unsaturated fatty acids other than that furnished by the fat being tested for its vitamin B-sparing action. This may have been a limiting factor in certain groups where low percentages of fats were fed. Where saturated fatty acid esters are the chief source of energy, the necessity for the inclusion of unsaturated fatty acids containing more than one double bond has been indicated (Evans

and Lepkovsky, '32 e and f). These essential unsaturated acids were adequately provided in all of our later experiments by the feeding of 0.20 ml. of U. S. P. linseed oil per rat daily (tables 5, 6, 7, and fig. 2).

Also in our experiments the autoclaved yeast was weighed separately and fed in uniform daily dosage to all the rats in a comparison series. Therefore, variations in food consumption did not result in variations in the intake of autoclaved yeast. The early work in our laboratory indicated that considerable variation in the results could be produced by varying the level of autoclaved yeast (Salmon and Guerrant, '30). This has been confirmed by the more recent work of Evans and Lepkovsky ('34 a) and by the results reported in this paper. The increase in growth and in the time required for onset of beriberi which results from increasing the level of autoclaved yeast, has been attributed to an increase of vitamin G by Evans, Lepkovsky and Murphy ('34 a). We are not inclined to consider this an adequate explanation of the effect. Our data in figure 1 show that adding 10 mg. of vitamin B solid was more effective than increasing the autoclaved yeast from 250 mg. to 500 mg. Moreover, adding 10 mg. of vitamin B solid to the 500 mg. allowance of autoclaved yeast again increased the rate of growth. It is evident that there was a marked stimulation of growth by some heat labile factor carried by the vitamin B solid. It is again shown in table 5 that increasing the level of autoclaved yeast from 250 mg. to 500 mg. when the low fat diet was being fed, prolonged the time required for the development of beriberi an average of 18 days. The effect is, therefore, not limited to a fat containing diet.

These results seem to us to indicate that the autoclaved yeast still contains traces of vitamin B. The effect of such traces is apparent even when a low fat diet is being fed but is accentuated by a high fat diet.

The data in this paper do not lend support to the view that a high protein intake is essential for the alleviation of vitamin B deficiency by fat. In the diets containing 40% or more of

fat, the protein content of the diets was increased in proportion to the increase in energy resulting from the substitution of fat for carbohydrate. In the diets containing esters equivalent to 23% of fatty acid, the casein was kept constant at 18%. The results reported in table 5 show that with this normal level of protein the effect of the esters was very pronounced.

The efficiency of an ester of the fatty acids in alleviating vitamin B deficiency in the rat appears to be related to the length of the carbon chain of the acid which the ester contains. In our experiments the efficiency was maximum at the 8-carbon acid. It decreased in each direction from this point until acetic on the one end and palmitic and stearic on the other end of the series were not effective at the 23% level. In fact the onset of beriberi was hastened by the replacement of sucrose with these acids. The odd carbon acids were slightly irregular but they followed the same trend as the even carbon acids.

Only in the results from the esters of palmitic and stearic acids was there evidence that decrease in efficiency might be attributed in part to poor absorption of the long chain compounds. The effectiveness of other members of the series as measured by the amount of growth produced, began to decrease, however, before there was any appreciable decrease in absorption. Moreover, oleic glyceride which was absorbed as well as lauric and myristic glycerides produce much less growth than either of these. Also acetic acid which was almost completely absorbed ranked very low.

The data on absorption or retention of the food fat indicate no impairment of fat digestion as a result of vitamin B deficiency. In the light of this it would seem that the reduction in pancreatic lipase activity in vitamin B deficient rats reported by Sure, Kik and Buchanan ('35) is not significant.

Despite differences in methods our results agree quite well with the major findings of Evans and Lepkovsky. We have studied a greater number of acids and have found a closer relationship between the length of the carbon chain of the

saturated acids and their effectiveness. Our data do not support the conclusions of the above investigators that the solid fats are more effective than the liquid fats. Furthermore, our results show that a high intake of vitamin G (autoclaved yeast) and of protein is not requisite to the demonstration of the improvement of vitamin B deficient diets by esters of certain fatty acids.

The results of Kemmerer and Steenbock ('33) do not necessarily conflict with the above results. It is evident that the fat diets which have been used do not completely obviate the necessity for vitamin B. It seems reasonable to expect that rats would first deplete their available body stores of vitamin B and then depend upon fatty acids to meet the nutritive emergency. In our experiments the esters of caproic and caprylic acids apparently cured cases of spastic beriberi. Presumably the usable stores of vitamin B were exhausted and fatty acid appeared to have substituted for this factor. This indicates that conservation of previously stored vitamin B is not an essential part of the action of fatty acids in alleviating vitamin B deficiency in rats.

The action of fats and the different effects of the fatty acids in conditions of vitamin B deficiency in the rat, present a puzzling problem. The advantage of a high fat diet over a low fat diet seems to disappear when the diet contains adequate vitamin B and the essential unsaturated fatty acids. Moreover, the ranking of the esters of certain fatty acids over the natural fats, when the diet is deficient in vitamin B, is reversed when a sufficiency of this factor is supplied. It thus appears that the 'vitamin B sparing action' of these substances is not due to any superior general nutritive value of certain fatty acids but is in some way specifically related to the deficiency.

The suggestion of Whipple and Church ('36) that vitamin B plays a role in the synthesis of fat from carbohydrate is a possibility to be considered. However, Schrader ('34) has shown in this laboratory that vitamin B deficient rats receiving a carbohydrate diet have respiratory quotients averaging 1.26.

Although this falls short of conclusive proof of transformation of carbohydrate into fat, it suggests that such a change is being made. Furthermore, if the sole function of vitamin B were as a catalyst in the synthesis of fat, it should be possible to compose a diet which would meet the requirements of animals for fat and support normal growth without the presence of vitamin B. We have no evidence that this can be done.

The idea that vitamin B functions in the metabolism of carbohydrate has had numerous proponents. The beneficial effect of replacing carbohydrate with fat in a diet which is inadequate in vitamin B, tends to support this idea. However, no one has yet been able to locate definitely the stage in the intermediary metabolism which is dependent upon this factor. Our data contribute to this only in showing that a relatively large amount of lactic acid ester in the diet tends to delay rather than hasten the onset of beriberi in the rat. This may be interpreted as indicating that vitamin B is not specifically related to the degredation of lactic acid.

SUMMARY

High percentages of fat in vitamin B deficient diets increased the rate of growth and decreased the incidence of beriberi in rats. Coconut fat was the most effective natural fat tested.

The effectiveness of esters of single fatty acids in alleviating the symptoms of vitamin B deficiency in rats depended upon the length of the carbon chain of the fatty acid. The effectiveness was maximum at the 8-carbon acid and decreased in each direction from this point.

Spastic beriberi in rats was cured by the feeding of glyceryl caprylate or caproate.

The apparent nutritive value of fats and single acid esters in vitamin B deficient diets was not the same as in diets that contained adequate vitamin B.

Increasing the autoclaved yeast intake improved the rate of gain and delayed the onset of beriberi in rats receiving vitamin B deficient diets but the addition of a source of vitamin B was more effective than increasing the autoclaved yeast.

A high intake of protein or of vitamin G (autoclaved yeast) was not a requisite for the action of fat in alleviating the effects of a vitamin B deficient diet.

Determinations of the refractive index of the fatty acid fraction of fat from the brains and livers as well as magneto-optic determinations of caprylic and caproic acids from these fractions failed to show any differences that could be attributed to the effect of vitamin B deficiency.

The magneto-optic minima of formaldehyde and formic acid were found in the brains, livers and blood of rats used in this study.

The results are discussed in relation to those obtained in other laboratories.

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THE RELATION OF INGESTED CARBOHYDRATE TO THE TYPE AND AMOUNT OF BLOOD AND URINE SUGAR AND TO THE INCIDENCE OF CATARACT IN RATS[1]

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The discovery and repeated confirmation of the fact that lactose and galactose rations may cause cataract in rats has been followed in this laboratory by extensive investigations as to the nature of the metabolic disturbances involved. No gross tissue pathology other than that of the lens is evident in these animals and a few histological studies of the endocrine organs have failed to reveal any significant changes. Numerous workers have found abnormal blood and urine sugar levels after the ingestion of large amounts of lactose or galactose both in man and in animals. Cori and Cori ('28) explained the more severe galactosuria after the ingestion of galactose on the ground of more rapid absorption taking place when the delay due to hydrolysis was not involved. Harding et al. ('34) concluded that there is, proportionally, a much larger excretion of ingested galactose in the urine in the rat than in man, and that there appears to be a lower rate of conversion of galactose into its metabolites in the rat. The present studies into the nature of the blood and urine sugar of rats fed high levels of these two sugars have been made in connection with further observations on factors influencing the rate of development of lens opacities.

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Although high blood sugar values in rats rendered cataractous by lactose and galactose rations have been found repeatedly in this laboratory, and confirmed by Day ('36), several pertinent questions still remain to be answered. How much of this abnormally high blood sugar is glucose and how much is galactose? Does the speed of cataract development correlate with the degree of galactosemia? How may other combinations of carbohydrates affect the blood sugar level and the crystalline lens?

EXPERIMENTAL PROCEDURE

In the present study the general procedure and experimental rations used were similar to those previously reported by Mitchell and Dodge ('35) and by Mitchell ('35). The skeleton ration formula in which the various carbohydrates were substituted was as follows:

	%
Carbohydrate	70
Casein	15
Salt mixture (O. and M.)	4
Crisco	9
Cod liver oil	2

Records of body weight, food intake, and cataract development have been kept systematically. Studies of blood and urine have been made on rats fed on rations containing 62 and 70% lactose, 25 and 35% galactose, 35% fructose, 35% xylose and 70% starch. Some preliminary observations have been made with Insulin-protamine injection in rats on the cataract producing 25% galactose ration. Four strains of rats have been studied on various cataract producing rations in an effort to determine whether differences in cataract susceptibility reported by Mitchell ('36) might be correlated with differences in blood or urine sugar levels. The rats used routinely were from the Battle Creek (B.C.) colony and the other strains studied were from the Johns Hopkins (J.H.), the Wistar (W.) and the local colonies (M.S.C.).

Blood sugar. Blood samples of 0.1 cc. (or 0.05 cc.) were drawn from the tail and blood sugar determinations made by

the Benedict micro-colorimetric method.[2] A free flow of blood was obtained by warming the tail in warm water before bleeding. There were practical difficulties in attempting to take blood samples from very young rats, but it was desirable to study the blood sugar during the period of cataract development if possible. Since cataracts developed in rats after 10 to 20 days on galactose rations (between the fortieth and fiftieth day of age) it was not always possible to obtain a long series of blood sugar determinations before the cataract developed. In the case of rats on lactose rations, lens changes were more delayed and there was no difficulty in obtaining a series of blood samples during the period of cataract development.

Blood sugar determinations were made under as nearly standard conditions as possible. A known amount of food (usually 3 gm.) was given early in the morning following a 16-hour fast and left in the cage for 1 hour. Usually the entire amount of food was eaten and most of it during the early part of the hour. Blood samples were taken ½ hour later presumably when the blood sugar level would be close to the maximum. Day ('36) found the peak somewhere between 1 and 2½ hours.

Various devices for differentiating glucose and galactose in blood have been investigated but the simplest and most satisfactory for routine procedure seemed to be a yeast fermentation. The Benedict micro-colorimetric method provided sufficient supernatant fluid after precipitation of proteins for the determination of the total sugar, leaving a remainder of slightly less than 3 cc. for yeast fermentation. Preliminary tests showed that glucose in the concentrations encountered in blood filtrates completely fermented in 30 to 40 minutes, while only a trace of added galactose was lost in that time. The fermentation procedure was standardized as follows: 2 cc. of a 10% baker's yeast suspension was centrifuged for 15 minutes, the supernatant fluid discarded, and the tube

[2] Blood sugar determinations were made by Vernon K. Watson and Katherine F. O'Brien.

wiped as dry as possible with a cotton swab. The 3 cc. of solution to be fermented was then mixed with the yeast cells in the centrifuge tube and allowed to stand at room temperature for 40 minutes with occasional stirring. After centrifuging, the second sugar determination was made upon the supernatant fluid. The reducing sugar present after fermentation was assumed to be galactose since control tubes containing glucose gave negative results and no other slowly fermenting sugar could logically be present in the blood of rats fed on starch and galactose rations.[3]

Attention is called to the fact that this fermentation method is only approximate, since the moisture retained by the yeast cells necessarily dilutes the solution to be fermented and, consequently, there is always a slight reduction in the sugar concentration due to this dilution irrespective of any fermentation, but no correction has been made to compensate for it. More prolonged centrifuging to pack the yeast cells harder and reduce the moisture content tended to reduce this error in the more recent determinations.

Urinary sugar. Determinations of total and nonfermentable sugar were made on 24-hour urine specimens collected in metabolism cages designed for the purpose. Most satisfactory specimens were obtained when the rats were fasted during the collection period. When food was allowed in the metabolism cage the danger of food contamination was present in spite of every precaution. The volume of urine from very young rats was so small as to involve a large error due to evaporation and accumulation on the sides of the cages and funnels. Nevertheless, an indication of the type of urinary sugar was determined when the volume obtained was sufficient for the fermentation procedure. Since cataracts developed so early on the galactose rations, it was not expedient

[3] Galactose was obtained from the SMA Corporation of Cleveland, Ohio. The specific rotation of this sugar is 80.0 as compared with a theoretical value of 80.5 for pure galactose. The ratio of galactose to glucose required to reduce a given amount of Benedict's quantitative solution is 1.18: 1. No corrections have been made in our data for this difference in reducing power—all results are recorded as for glucose.

to maintain the animals on the expensive diets over long periods of time. Consequently, there were but few urinary sugar determinations in the galactose groups except where cataract development was delayed as in the Johns Hopkins and Wistar groups.

Rats remained on the lactose rations much longer and there was no difficulty in obtaining sufficient volume of urine for sugar analysis. Another difficulty was encountered in this group, however, because of accompanying diarrhea. Metabolism cages equipped with fine wire gauze a few inches beneath the coarse wire mesh floor of the cage held back all formed stools, and glass wool in the glass funnel retained smaller debris, but fluid stools could not be separated successfully from the urine. Where serious fecal contamination was evident the specimen was discarded, but where a small amount of sediment only was present the urine was clarified by centrifuging. The Myers ('24) micro-modification of the Benedict titration method was used for urinary sugar determinations and the fermentation procedure was carried out as described in a previous paragraph.

DISCUSSION

In attempting to evaluate and compare the blood or urine sugar figures obtained in this study, one is impressed with the wide variations and some inconsistencies. These are to be expected, however, under the conditions of the experiment, but the number of figures obtained in the several series here reported and the statistical treatment of the results give a strikingly significant and interesting picture.

Blood sugar. The observations summarized in table 1 show total blood sugar values well above normal in all groups on cataract producing rations. The apparent correlation of the susceptibility to cataract with the average blood sugar levels in the 35 and 25% galactose and the 70% lactose groups is striking, but its significance is lessened when one analyzes the figures for the individual animals in any one of these groups. The most rapid cataract development does not

TABLE 1

Blood sugar values and incidence of cataract

CARBOHYDRATE IN THE RATION	BREED	NUMBER OF RATS	TOTAL SUGAR		NONFERMENTABLE		FERMENTABLE		CATARACT [1]	
			Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Number of determinations	Mean with probable error	Incidence	Development
				mg./100 cc.		*mg./100 cc.*		*mg./100 cc.*	%	*days*
35 G + 35 S	B.C.	15	34	274±6.1	28	172±6.7	28	102±4.0	100	16
35 G + 35 S	W.	14	31	264±3.4	17	185±5.3	17	79±4.9	75	35
35 G + 35 S	J.H.	14	29	275±5.6	14	195±5.9	14	86±4.7	90	30
25 G + 45 S	B.C.	44	70	246±3.0	48	141±3.7	48	113±2.8	94	22
25 G + 45 S + I-p (Lilly)	B.C.	7	13	252±8.4	8	170±5.5	8	103±4.9	100	22
70 L	B.C.	16	34	155±3.4	29	72±3.2	29	87±3.1	72	47
62 L	B.C.	15	29	164±4.9	15	73±3.1	15	92±5.1	29	66
35 X + 35 S	B.C.	2	8	119±5.6					0	..
35 F + 35 S	B.C.	2	9	103±5.8					0	..
70 S	B.C.	26	51	112±1.8					0	..

TABLE 2

Urinary sugar values and incidence of cataract

				mg./24 hours		%				
35 G + 35 S	W.	16	41	93±8.0	16	93±0.7			75	35
35 G + 35 S	J.H.	12	26	61±7.2					90	30
70 L	B.C.	37	95	63±5.3	59	86±1.0			76	39
70 L	M.S.C.	10	40	53±4.3	53	86±3.8			10	56
62 L	B.C.	12	23	35±2.9	12	82±1.5			29	66
70 S	All breeds	35	56	0 (qual.)					0	..
		12	28	7±0.6					0	..

[1] Cataract data relate to the litters included in this study.

G = galactose, S = starch, L = lactose, X = xylose, F = fructose, I-p = Insulin-protamine (Lilly).

parallel the highest blood sugar levels nor do resistant individuals show a consistently lower blood sugar. It is conceivable that the duration of galactosemia may be quite as pertinent as its degree.

The question of the influence of breed upon galactose tolerance was a logical outcome of observations reported by Mitchell ('36) on breed difference in susceptibility to cataract. Average blood sugar values on three strains of rats fed the 35% galactose ration were strikingly similar, thus affording no explanation to account for the observed differences in cataract development. One Wistar litter which proved to be unusually resistant to cataract did show the lowest blood sugar of the rats of the same strain, but such litter variance was not consistent in other groups. In the Johns Hopkins strain the black-hooded rats developed cataract in one-third the time required for the buff-hooded, whereas the latter group showed galactosemia distinctly above the average for all rats of that strain. In none of these three strains were the blood sugar values as high or as variable as those reported by Day ('36) even though our observations included larger groups of animals. It is impossible to conclude, therefore, that the degree of galactosemia, as observed, is alone responsible for the developing lens opacities although there is undoubtedly a close relationship.

More interesting even than the level of the total blood sugar in the cataractous rats is the nature of this sugar. The difference between the total and the nonfermentable fraction (galactose) is assumed to be the blood glucose. Cori ('25) found that during the absorption of galactose there was no rise in the glucose content of the blood. Our present findings constitute a striking verification of this statement, although the conditions of the experiment were quite different. It is significant to compare the blood sugar of 112 mg./100 cc. in the starch control group with the fermentable fractions of the blood sugars in several of the lactose and galactose groups, which are all within the range of normal blood sugar figures. The fact that some of the latter are slightly less than normal

blood glucose figures may well be accounted for by the errors of experimental technic or may represent a true depression of the blood glucose level.

The nonfermentable fraction is in most cases responsible for the differences noted in total blood sugars in the respective ration groups. Thus it appears that the rapid absorption and slower utilization of galactose allows it to accumulate in the blood over and above the normal blood glucose which apparently is not greatly altered thereby. In fact it behaves like a soluble foreign body in the blood stream. The greater variability of the nonfermentable fraction is easily explained by differences in individual tolerance, by time of feeding, and by the total amount of food eaten. A nonfermentable sugar fraction was never found in the blood of starch-fed control rats.

A limited number of observations were made with other carbohydrate combinations; 35% fructose and 35% xylose, respectively, were substituted for a portion of the starch in the skeleton ration. When fructose was fed the blood sugar was normal and there were no eye changes. Xylose caused a slight rise in total blood sugar with a small nonfermentable fraction seeming to account for the increase. Ophthalmoscopic observations on the xylose group revealed some early and rather transitory lens changes which never progressed beyond this stage.

The lower incidence and slower development of cataract on lactose rations along with the usual diarrhea complication and retarded growth have resulted in curtailment of work with this sugar. The 62% lactose ration was employed with and without various additions (4, 6 and 8%) of calcium lactate. The original purpose was to observe whether excess calcium in a lactose ration would have any effect upon the development of lens opacities. These results were negative, but the calcium lactate tended to inhibit the diarrhea due to lactose and proved to be toxic at the higher levels. Blood sugar values under these conditions were entirely comparable to those obtained with the other high lactose ration and are of

interest here merely as further evidence of the partition of the sugar in the blood.

Consideration was given to the possibility of lowering the blood sugar to within normal limits while feeding galactose. Roe and Schwartzman ('32) and Mason and Turner ('35) have shown that insulin does not lower blood galactose in the human. Corley ('27) found that insulin did hasten the disposal of intravenously administered galactose in the rabbit. The possibility that the rat might respond differently could not be ignored. The problem of insulin shock and fluctuation of blood sugar level in a rat eating ad libitum eliminated regular insulin as a feasible agent. Some preliminary tests with the new Insulin-protamine (Lilly)[4] showed it to be slowly absorbed from the subcutaneous tissue of the rat. The action seemed to be prolonged for more than 24 hours and the maximum dose tolerated by young rats was 4 units administered every 48 hours. A limited number of observations were made on rats receiving a 25% galactose ration with graded doses of Insulin-protamine. No significant reduction in total blood sugar was observed and cataracts developed at approximately the same rate as in the control litter mates on the same ration without insulin. These findings would seem to confirm the observations of previous workers that insulin cannot lower blood galactose.

Urinary sugar. Both the type and amount of urinary sugar were determined on groups where this procedure was expedient. The comparative differences shown in table 2 for total sugar excreted in 24 hours are far less significant than for the blood sugar values because the size of the rat and the total food intake were not entirely comparable in the different groups. The severe diarrhea in young rats on lactose prevented the collection of satisfactory urine specimens at as early an age as was necessary in the galactose groups. Thus galactosuria was relatively much more severe on the galactose than on the lactose rations although the figures fail to emphasize this fact.

[4] Supplied through the courtesy of the Eli Lilly Company.

The type of sugar excreted was more significant than the total amount. The sugar remaining in the samples after 40 minutes yeast fermentation ranged from 82 to 93% of the total present before fermentation. Data are given in the table for four groups but other scattered determinations were invariable within the same range. The unavoidable dilution of the specimens in the course of the fermentation procedure and a possible decomposition of a trace of galactose are more plausible explanations of the 7 to 18% loss than the presence of an appreciable amount of fermentable sugar. Pure galactose solutions subjected to the same treatment showed similar losses in concentration.

Thus it may be concluded that galactose is the sugar responsible for both the high blood and high urine sugars observed in rats fed on lactose and galactose rations and must be the major etiological factor in this type of cataract.

SUMMARY

1. Blood and urine sugar studies have been made on rats fed on adequate rations containing 62 and 70% lactose, 25 and 35% galactose, 35% fructose, 35% xylose and 70% starch.

2. Determinations of total and nonfermentable sugar were made on both blood and urine specimens from the various ration groups.

3. Total blood sugar values were higher on galactose rations than on lactose but above normal in all animals on cataract producing rations.

4. The nonfermentable fraction of blood sugar was chiefly responsible for the differences in total blood sugar in the various groups, the fermentable fraction remaining more nearly constant and within the range of normal blood glucose.

5. Average total blood sugar values of three strains of rats fed on 35% galactose ration were strikingly similar, in contrast to the differences observed in susceptibility to cataract among the same groups.

6. A fructose-starch ration caused no hyperglycemia and no eye changes.

7. A xylose-starch ration brought about a slight elevation in blood sugar and some early transitory lens changes which did not progress beyond this stage.

8. Insulin-protamine (Lilly) failed to lower blood galactose or reduce the speed of cataract development on a 25% galactose ration.

9. A galactosuria of varying degrees was observed in all rats on lactose and galactose rations, relatively more severe in the latter and absent in starch-fed control groups.

10. It may be concluded that galactose is the sugar responsible for both the high blood and the high urine sugars observed in rats fed on lactose and galactose rations and must be the major etiological factor in this type of cataract.

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ANTITHYROGENIC ACTION OF CRYSTALLINE VITAMIN B [1]

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In a previous investigation Sure and Smith ('34) demonstrated that retardation of loss of weight can be secured in animals receiving toxic doses of pure crystalline thyroxin (Squibb's) by administration of a potent vitamin B concentrate.

The isolation of vitamin B(B_1) in pure crystalline form, supplied by Merck and Company, made possible a quantitative study of this problem. Various doses of thyroxin were given and an attempt was made to find the requirements of crystalline vitamin B to prevent loss of weight produced by hyperthyroidism. It was found that the efficiency of vitamin B in overcoming the toxicity of thyroxin depends largely on the source of the stable components of the vitamin B complex contained in the experimental ration. The results are summarized in tables 2 and 3.

Sixteen groups of rats in sets of four, litter mates of the same sex, were given graduated daily doses of thyroxin, 0.05 to 0.2 mg. with various daily doses of crystalline vitamin B, 1 γ to 160 γ daily. First, all animals were depleted on ration 1751, deficient only in the vitamin B complex. They were then transferred to ration 2345, deficient only in vitamin B, the antipellagric factor of which was furnished with 15% of autoclaved round steak, and dried at 100°C. This ration was supplemented with 4 drops of cod liver oil per animal per day.

[1] Research paper no. 430, journal series, University of Arkansas.

In each experiment of this series the first animal served as the negative control, that is, it received a diet deficient only in vitamin B. The second animal received the same diet and thyroxin. The third animal received the B deficient diet, thyroxin and crystalline vitamin B. The fourth rat served as the positive control, which received vitamin B but no thyroxin. The gain in weight of the positive control was taken as 100%, and the gains in weight, if any, of the other animals in the group were then calculated accordingly. The

TABLE 1

Composition of rations

	NUMBER OF RATIONS		
	1751	2345	3080
Casein	20[1]	20[2]	20[2]
Agar-agar	2	...	...
Salts no. 185	4	4	4
Butterfat	10	10	10
Autoclaved beef[3]	...	15	...
Autoclaved Northwestern yeast[4]	...	...	15
Dextrin	64	51	51
	100	100	100

[1] Washed with acidulated water for 10 days.

[2] Washed for 10 days with acidulated water and extracted thoroughly with 25% alcohol.

[3] Autoclaved for 6 hours at 20 pounds pressure at the natural pH of the beef (round steak).

[4] Dehydrated baker's yeast supplied by the Northwestern Yeast Company, Chicago, Illinois. This yeast was autoclaved for 6 hours at 15 pounds pressure at the pH of 6.8.

incidence of protection was figured from the changes in body weight. For instance, if a positive control gained during the experimental period of 30 days, 24 gm., and if the litter mate that received thyroxin and vitamin B gained 12 gm. during this interval, that animal was considered as receiving 50% protection. Losses of weight were designated as zero protection.

It will be noted that on a daily dose of 0.05 mg. of thyroxin, 3 to 5 γ of B afforded 40 to 48% protection and 10 γ of B

TABLE 2

Antithyrogenic action of crystalline vitamin B on ration 2345

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE CRYSTALLINE VITAMIN B_1	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	PER CENT PROTECTION
		days	*mg.*	*γ*	*gm.*	*gm.*	*gm.*	
2991	♂-NC	17	....	...	74	56	— 18	0
	L♂		0.05	...	88	64	— 24	0
	R♂		0.05	1	89	63	— 26	0
	D♂-PC		....	1	70	87	+ 17	100
2992	♀-NC	17	....	...	74	58	— 16	0
	L♀		0.05	...	74	50	— 24	0
	R♀		0.05	3	75	83	+ 8	48
	D♀-PC		....	3	62	79	+ 17	100
2993	♂-NC	14	....	...	63	39-D	— 24	0
	L♂		0.05	...	65	51	— 14	0
	R♂		0.05	5	55	65	+ 10	40
	D♂-PC		....	5	70	95	+ 25	100
2994	♀-NC	35	....	...	64	55	— 11	0
	L♀	10	0.05	...	73	50	— 23-DC	0
	R♀	35	0.05	10	62	113	+ 51	137
	D♀PC	35	....	10	44	81	+ 37	100
2984	L♂-NC	23	....	...	66	59	— 7	0
	R♂	12	0.1	...	68	49	— 19-DC	0
	D♂	23	0.1	1	59	55	— 4	0
	DR♂-PC	23	....	1	60	82	+ 22	100
3004	D♀-NC	53	....	...	70	60	— 10	0
	R♀	15	0.1	...	75	61	— 14-DC	0
	DL♀	53	0.1	3	70	62	— 8	0
	L♀-PC	53	....	3	67	95	+ 18	100
3006	L♀-NC	22	....	...	68	59	— 9	0
	R♀	11	0.1	...	68	54	— 14-DC	0
	D♀	40	0.1	8	65	78	+ 13	18
	DR♀-PC	40	....	8	63	133	+ 70	100
3007	♂-NC	37	....	...	80	66	— 14	0
	L♂	18	0.1	...	80	62	— 18-DC	0
	D♂	39	0.1	10	77	91	+ 14	18
	DL♂-PC	39	....	10	75	152	+ 77	100

TABLE 2—*Continued*

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE CRYSTALLINE VITAMIN B_1	INITIAL WEIGHT	FINAL WEIGHT	CHANGE IN WEIGHT	PER CENT PROTECTION
		days	*mg.*	*γ*	*gm.*	*gm.*	*gm.*	
3008	♀-NC	21	....	...	90	64	— 26	0
	D ♀	29	0.1	...	90	67	— 23	0
	DL ♀	40	0.1	20	86	110	+ 24	42
	L ♀-PC	40	....	20	78	135	+ 57	100
3029	R ♀-NC	15	....	...	66	56	— 10	0
	D ♀		0.2	...	72	51	— 21	0
	L ♀		0.2	3	58	47	— 9	0
	♀-PC		....	3	83	83		100
3030	♂-NC	21	....	...	83	71	— 8	0
	L ♂	7	0.2	...	77	55	— 22-DC	0
	R ♂	13	0.2	5	65	49	— 16	0
	D ♂-PC	13	....	5	73	85	+ 12	100
3039	♀-NC	22	....	...	78	61	— 17	0
	L ♀	7	0.2	...	68	48	— 20-DC	0
	R ♀	15	0.2	8	70	52	— 18	0
	D ♀-PC	15	....	8	66	71	+ 5	100
3041	♂-NC	17	....	...	72	69	— 3	0
	L ♂	8	0.2	...	71	52	— 19-DC	0
	R ♂	11	0.2	10	65	54	— 11	0
	D ♂-PC	11	....	10	69	73	+ 4	100
3042	♀-NC	21	....	...	72	56	— 16	0
	L ♀	5	0.2	...	62	47	— 15-DC	0
	R ♀	10	0.2	20	67	56	— 11	0
	D ♀-PC		....	20	57	71	+ 14	100
3043	♀-NC	47	....	...	63	41	— 22	0
	L ♀	7	0.2	...	62	43	— 19-DC	0
	R ♀	11	0.2	80	74	74		0
	D ♀-PC	11	....	80	68	84	+ 16	100
3054	♀-NC	53	....	...	70	40	— 30-D	0
	L ♀	12	0.2	...	75	43	— 28-D	0
	R ♀	28	0.2	160	69	83	+ 14	32
	D ♀-PC	28	....	160	67	111	+ 44	100

NC = negative control; PC = positive control; D = died; DC = dying condition.

TABLE 3

Antithyrogenic action of crystalline vitamin B on ration 3080

EXPERIMENT NO.	ANIMAL MARKING AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE N. W. YEAST	DAILY DOSE VITAMIN B_1	INITIAL WEIGHT	FINAL WEIGHT	CHANGE OF WEIGHT	PER CENT PROTECTION
		days	*mg.*	*mg.*	*γ*	*gm.*	*gm.*	*gm.*	
3140	R ♂-C	42	...	500	...	100	214	+ 114	100
	L ♂		0.2	500	...	100	159	+ 59	52
	♂		0.2	...	10	117	134	+ 17	15
3141	DR ♀-C	42	...	500	...	111	180	+ 69	100
	DL ♀		0.2	500	...	107	193	+ 86	124
	D ♀		0.2	...	30	99	164	+ 65	94
3142	R ♀-C	42	...	500	...	118	186	+ 68	100
	L ♀		0.2	500	...	122	166	+ 44	65
	♀		0.2	...	50	110	160	+ 50	74
3143	DR ♂ C	42	...	500	...	122	241	+ 119	100
	DL ♂		0.2	500	...	118	216	+ 98	82
	D ♂		0.2	...	100	100	188	+ 88	74
3144	R ♂-C	39	...	500	...	95	207	+ 112	100
	L ♂		0.2	500	...	98	188	+ 90	80
	♂		0.2	...	10	92	154	+ 62	53
3146	R ♀-C	39	...	500	...	99	165	+ 66	100
	L ♀		0.2	500	...	101	186	+ 87	132
	♀		0.2	...	50	99	170	+ 71	108
3147	DR ♂-C	39	...	500	...	76	221	+ 145	100
	DL ♂		0.2	500	...	90	202	+ 112	77
	D ♂		0.2	...	100	95	188	+ 93	64
3111	D ♂-C	49	...	500	...	80	256	+ 176	100
	R ♂		0.2	500	...	69	229	+ 160	94
	L ♂		0.2	...	300	88	214	+ 126	71
	♂		0.2	...	100	90	230	+ 140	80

C = control.

produced a greater gain of body weight than the positive control. On a 0.1 mg. daily dose of thyroxin, however, as much as 20 γ of B allowed only 42% protection. The daily dose of 0.2 mg. of thyroxin is so toxic to animals on ration 2345 that even 80 γ daily of B produced no antithyrogenic action, and even on 160 γ of vitamin B, only 32% protection was secured.

Numerous experiments in this laboratory have demonstrated that considerably greater growth is obtained with graduated daily doses of crystalline vitamin B, using autoclaved Northwestern yeast, regardless of whether it was autoclaved at 15 or 20 pounds pressure, than with autoclaved beef, as a source of the stable components of the vitamin B complex. Since autoclaved beef to the extent of 15% in the ration supplies an abundance of the rat antipellagric factor, the dried baker's yeast must be furnishing a stable factor that is deficient in the autoclaved beef. Our observations, therefore, substantiate the claims of György ('35), Harris ('35), Chick and co-workers ('35), and of Elvehjem and associates ('36), that vitamin G (or B_2) is a complex consisting of at least two dietary essentials.

A series of experiments of eight groups were then investigated on a 0.2 mg. daily dose of thyroxin, with graduated daily amounts of crystalline vitamin B, 10 to 300 γ, on ration 3080, which contained 15% dried autoclaved baker's yeast (Northwestern) as a source of the vitamin G complex. In this study the animal of each group receiving a daily dose of 500 mg. of untreated Northwestern yeast, without thyroxin additions, was considered as the positive control. The graduated doses of crystalline vitamin B were then compared with 500 mg. daily of Northwestern yeast, in the presence of a daily allowance of a toxic dose of 0.2 mg. thyroxin. While there is considerable variation in the extent of antithyrogenic efficiency produced by the various doses of vitamin B, the fact that 64 to 94% protection has been secured with such small daily amounts as 30 to 100 γ of vitamin B, establishes the value of this vitamin as an antithyrogenic agent in experimental

hyperthyroidism. Furthermore, the greater efficiency of crystalline vitamin B on ration 3080 than on ration 2345, is due to the former ration furnishing a stable dietary factor deficient in the latter ration. We also found that the efficiency of rice polishings as an antithyrogenic agent is considerably enhanced on ration 2345 after it is supplemented with a daily allowance of 100 mg. of Lilly liver extracts (used in pernicious anemia) which is abundant in what was heretofore referred to as vitamin G, but which must be now regarded as a complex. Our interpretation is that the liver extracts supply a hitherto unrecognized stable factor of the vitamin G complex, probably synonymous with the new dietary factor of Elvehjem and associates ('35).

SUMMARY

After the stable components of the vitamin B complex have been provided for, which may be furnished by autoclaved Northwestern dehydrated baker's yeast, pure crystalline vitamin B_1 becomes an excellent antithyrogenic agent in daily doses of 30 to 100 γ to counteract the toxicity of as high a daily dose as 0.2 mg. of thyroxin.

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INFLUENCE OF HYPERTHYROIDISM ON VITAMIN A RESERVES OF THE ALBINO RAT [1]

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That various foodstuffs may exert a protective influence in experimental hyperthyroidism has been pointed out by Abelin ('30), Abelin, Knochel and Spichtin ('30). Von Euler and Klussmann ('32) observed that rats on a diet deficient in vitamin A and injected with thyroxin, suffered loss of weight less readily when also receiving liberal amounts of carotene. In 1935 Abelin reported that vitamin A can reduce the high basal metabolic rate produced in rats given toxic doses of thyroxin; also, that vitamin A administered as carotene will restore the reduced glycogen content of the liver in hyperthyroidism in the rat. Neither von Euler nor Abelin, however, was able to prevent the large losses of body weight caused by administration of large doses of thyroxin.

Since we found that vitamin B(B_1) acts as an efficient antithyrogenic agent only in the presence of an abundance of the stable components of the vitamin B complex (Sure and Buchanan, '37), we have introduced a diet composed largely of dried milk, as follows: Skimmed milk powder, 50; agar-agar, 2; salts no. 185, 4; ferric citrate, 0.2; butterfat, 10; and dextrin, 37.8. This ration was supplemented with 4 drops of cod liver oil per animal per day; in this study a daily dose of 0.2 mg. of thyroxin was employed. As a source of vitamin B we used Lilly vitamin B concentrate prepared for parenteral clinical use, kindly furnished by Mr. Rhodehamel of the Eli

[1] Research paper no. 431, journal series, University of Arkansas.

Lilly Research Laboratories. This concentrate was supplied in ampules in liquid form, each cubic centimeter containing 150 Sherman-Chase vitamin B units. According to Waterman and Ammerman ('35) one Sherman-Chase unit is equivalent to 2.5 γ crystalline vitamin B. The units of vitamin B introduced by the Lilly concentrate were, therefore, translated in γ vitamin B.

The results are summarized in table 1. We were surprised to find that on this dietary regime, which furnished the same amounts of butterfat and cod liver oil as sources of vitamin A as ration 2345 (for composition see preceding paper), three animals, after showing excellent responses to Lilly vitamin B concentrate in antagonizing the toxicity of thyroxin, developed marked cases of xerophthalmia and died. One animal suffered with labored respiration, typical in the terminal stages of vitamin A deficiency.

It is true that a ration containing large amounts of dried milk powder to which fats have been added suffers vitamin A losses on standing (Sure, '29), but since this diet (3109) without thyroxin additions, furnished ample amounts of vitamin A for excellent growth, as is evident from table 1, the daily dose of 0.2 mg. thyroxin must have produced a depletion of the vitamin A reserves on the animals that succumbed with accompanying severe eye lesions after excellent responses have been obtained to vitamin B additions. In this respect we concur with the observations of Abelin ('35).

In six groups of rats an attempt was made to counteract the toxicity of a 0.2 mg. daily dose of thyroxin, when crystalline vitamin B proved ineffectual on ration 2345 (which supplied the stable components of the vitamin B complex in the form of autoclaved beef) with massive doses of carotene in oil, furnishing 500 units of vitamin A daily, given as 10 drops three times daily. Such massive doses of vitamin A produced no changes in body weight and showed no antithyrogenic value. Vitamin A even in massive doses cannot, therefore, replace the essential stable component furnished by autoclaved dried baker's yeast, and which is deficient in autoclaved beef.

TABLE 1

Antithyrogenic action of Lilly vitamin B concentrate on ration 3109

EXPERIMENT NO.	ANIMAL MARKINGS AND SEX	EXPERIMENT PERIOD	DAILY DOSE THYROXIN	DAILY DOSE LILLY B_1 CONCENTRATE	DAILY DOSE N. W. YEAST	INITIAL WEIGHT	FINAL WEIGHT	CHANGE OF WEIGHT	PER CENT OF PROTECTION	XEROPHTHALMIA[1]
		days	*mg.*	*γ*	*mg.*	*gm.*	*gm.*	*gm.*		*day*
3109	D ♂-PC	57	...	...	500	132	263	+ 131	100	27
	R ♂	57	0.2	...	500	123	180	+ 57	43	27
	♂	26	0.2	15	...	117	156	+ 39	30	27
		57				117	98	— 19-D	0	
	L ♂	31	0.2	30	...	109	177	+ 68	52	27
		43				109	138	+ 29-D	22	
3112	D ♀-PC	35	...	...	500	112	160	+ 48	100	31
	R ♀	35	0.2	...	500	114	156	+ 42	88	
	♀	35	0.2	75	...	122	138	+ 16	33	
	L ♀	35	0.2	150	...	110	139	+ 29	60	
3113	D ♀-PC	56	...	...	500	122	195	+ 73	100	
	R ♀	56	0.2	...	500	132	214	+ 82	112	31
	♀	56	0.2	150	...	117	156	+ 39	53	
	L ♀	26	0.2	225		117	136	+ 19	23	
		34				117	97	— 20-D	0	27
3127	D ♂-PC	35	...	...	500	128	238	+ 110	100	
	R ♂	35	0.2	...	500	137	212	+ 75	68	
	♂	35	0.2	225	...	138	208	+ 70	63	
	L ♂	35	0.2	300	...	140	202	+ 62	57	

[1] The xerophthalmia was accompanied by labored respiration.

PC = positive control; D = died.

SUMMARY

A diet containing 50% dried skimmed milk powder furnishes a sufficient amount of the stable components of the vitamin B complex, so that excellent responses are obtained to pure crystalline vitamin B as an antithyrogenic agent in experimental hyperthyroidism. Such a ration, however, containing 10% butterfat and supplemented with 4 drops of cod liver oil per animal per day, does not provide a sufficiency of vitamin A to counteract the rapid catabolism produced by a daily administration of 0.2 mg. of thyroxin.

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THE VITAMIN G[1] CONTENT OF SOME FOODS

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The experiments reported in this paper were carried out before it was established by the work of several investigators that a) what was hitherto called vitamin G is composed of at least two factors; namely, flavin and vitamin B_6, both these factors being necessary in the diet to permit normal growth and to maintain a normal condition of the skin in the rat (György, '35 a; Chick, Copping and Edgar, '35; Harris, 35; Copping, '36 a), b) neither flavin nor vitamin B_6 are effective in the prevention or treatment of human pellagra (György, '35 b; Spies and Chinn quoted by Birch, György and Harris, '35; Dann, '36), and c) assays employing the Bourquin-Sherman ('31) or the Munsell ('31) vitamin G-deficient diets measure the flavin content of a substance under test (Booher, Blodgett and Page, '34; Bisbey and Sherman, '35; Copping, '36 b; Day and Darby, '36). Some time before these facts were established, we determined the vitamin G content of various foods with a view toward locating inexpensive food sources which it was hoped would prove effective in combatting human pellagra in the south. In these tests, we employed both the Bourquin-Sherman and the Munsell vitamin G deficient diets which as stated above serve to measure the flavin content of a test material. Although we later learned that measurement of the flavin content is of no apparent value in determining the effectiveness of a food in the prevention or

[1] In this paper, vitamin G refers to the factor flavin as determined by the Bourquin-Sherman vitamin G-deficient diet ('31).

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treatment of human pellagra, nevertheless these assays should be placed on record. Recent work indicates that flavin besides being a definite growth-promoting substance and concerned in the maintenance of a normal skin condition, also plays a role in food utilization and stimulation of the appetite (Griffith, '36).

The Bourquin-Sherman vitamin G deficient diet carries an 80% ethyl alcohol extract of whole wheat in such proportion that 100 gm. of the diet contain the extract of 50 gm. of whole wheat to serve as a flavin-deficient source of all growth factors of the vitamin B complex. The Munsell diet carries 30% white corn in place of the alcoholic wheat extract to serve the same purpose. Employing these two vitamin G deficient diets or modifications of them, several investigators (Booher, Blodgett and Page, '34; György, '35 a; Copping, '36 a, b; Day and Darby, '36) have demonstrated that flavin brings about renewal of growth when added to the diet of rats depleted on these rations. Elvehjem, Koehn and Oleson ('36) have recently reported their inability to produce growth in rats on a purified ration supplying vitamins B, B_2, B_4, B_6 and flavin unless another distinct factor which they have called the alcohol-ether precipitate factor is added to the diet. These facts taken together indicate that the above-mentioned vitamin G deficient diets contain all the factors needed to supplement flavin in exerting its growth-promoting effect.

That the Bourquin-Sherman diet, however, contains a limited amount of some factor or factors other than vitamin B and B_6 is evidenced by the findings of Booher, Blodgett and Page ('34) and Copping ('36 b). These workers employed multiples of the regular amount of alcoholic wheat extract used in the diet. Taking a low level of their flavin preparation as a supplement, Booher and co-workers ('34) found that "where growth is limited to 6.0 gm. per week, an increase in the level of wheat extract is of no advantage over our usual vitamin G deficient diet." However, when higher levels of flavin were fed yielding higher growth rates, the regular ration permitted only about 73 to 76% of the gain made by

animals on the reinforced diets. Copping ('36 b) worked only with a high rate of gain. When data for rats showing the same food intake are chosen from Copping's protocols, results similar to those of Booher and co-workers are secured. These findings can be interpreted to indicate that when flavin is the test material, the Bourquin-Sherman diet contains an adequate amount of some factor or factors (other than vitamin B_1 and B_6) for a rate of gain limited to 6.0 gm. weekly, but an insufficient amount when higher rates of gain are involved. Of course, when natural foods are assayed it is possible that data secured with higher growth rates may be reliable for calculation of vitamin potency, if it is known that the test food contains an abundance of the limiting factor or factors in the Bourquin-Sherman diet. In practically all the assays on natural foods reported in this paper, we have calculated the vitamin potency from data where the growth rate was limited to a gain of 3.0 to 6.0 gm. per week.

Several investigators (György, '35 a; Chick, Copping and Edgar, '35; Copping, '36 a and b) have shown that there are at least two types of skin lesions occurring in the rat, the 'specific' or 'acrodynia-like' type resulting from a deficiency of vitamin B_6, while the 'non-specific' type is brought about by a lack of flavin in the diet. Copping was consistently able to produce the 'non-specific' type of lesion in rats depleted on the Bourquin-Sherman diet and to bring about a cure of this skin derangement and restoration of growth by the administration of flavin.

From all the facts presented above it is clear, therefore, that the Bourquin-Sherman ration by virtue of a flavin deficiency permits the development of the 'non-specific' type of skin lesion and brings about cessation of growth in the rat, flavin curing the skin disorder and restoring growth. These facts are also presented here to show that assays employing the Bourquin-Sherman or Munsell vitamin G deficient diets serve to measure the flavin content of a test substance. At the time our assays were carried out, pure flavin was not available to us so that it was not possible to carry out parallel

feeding experiments with flavin in direct comparison with the foods which we tested. Our results, therefore, are based on the reliability of the feeding technic which we employed. Day and Darby have recently reported that 4 γ of flavin are equivalent to one Bourquin-Sherman vitamin G unit. However, until the growth-promoting property of pure flavin has been quantitatively established with the Bourquin-Sherman diet, it is desirable to express the flavin potency of foods in terms of Bourquin-Sherman units of flavin rather than in terms of weight of flavin. In our assays, therefore, we report the vitamin potency on this basis. Bisbey and Sherman likewise have used the Bourquin-Sherman diet to measure flavin values.

EXPERIMENTAL PART

In looking about for sources of vitamin G, our attention was first drawn to cottonseed meal and soy beans, both inexpensive and abundant foodstuffs. Several investigators have reported that when cottonseed meal or flour (Osborne and Mendel, '17 a; Richardson and Green, '17; Hunt, '32) or soy beans (Daniels and Nichols, '17; Osborn and Mendel, '17 b; Salmon, '27; Wilkinson and Nelson, '31; Rittenger and Dembo, '32) constituted the only source of the 'vitamin B complex' in the diet, normal growth was permitted in the rat. These findings indicated that these foods are probably good sources of vitamin G and prompted us to assay these foods for their content of this vitamin. Since we were at the same time interested in knowing whether milk produced in a pellagrous area was as potent as that produced in other regions, we assayed a sample of dried whole milk produced in South Carolina. For comparative purposes, we also tested a sample of dried brewer's yeast used by the South Carolina State Board of Health in combatting pellagra. In addition to these tests, the solubility of vitamin G (as present in cottonseed meal) in 50% ethyl alcohol and the influence of pressure cooking on the vitamin G content of both cottonseed meal and soy beans was determined. In a preliminary experiment, the Munsell ('31) flavin-deficient diet was employed in assaying

the above-mentioned foods. Comparison of the results obtained was then made with the results of subsequent assays in which the Bourquin-Sherman ration was used.

Series I

The rats used in this preliminary series and in the following series of experiments were bred in our laboratory on a stock ration described elsewhere (Levine, Remington and Culp, '31). When the average body weight of the rats in each litter reached about 60 gm., they were then placed on the flavin-deficient ration described by Munsell. A total of forty-four rats were employed in the feeding tests. For about 2 weeks the animals gained in weight after which there was a tendency toward stationary or declining weight. After the body weight had become constant or had begun to decline for a period of 1 week (usually at the end of 21 to 25 days), various supplementary foods were given to the animals placed in individual cages equipped with raised wire screen bottoms. In some cases, mild alopeceia appeared at this time.

The supplements fed were cottonseed meal, soy beans (Biloxi variety), dried whole milk powder and dried brewer's yeast. The cottonseed meal and soy beans were purchased in the open market. The supplements were fed daily to each rat for a period of 8 weeks. The results obtained in our feeding tests are given in table 1. The negative control rats on the unsupplemented basal ration made an average gain of + 10 gm. in body weight during the curative period. This finding agrees closely with that of Munsell and of Douglass and co-workers ('34) on the same ration. The Munsell diet is, therefore, not as free from flavin as the ration of Bourquin and Sherman which, according to growth curves given by these investigators and our own findings, permits an average loss of — 4.0 gm. during the 8 weeks test period. It is customary to express the vitamin G content of foods (Sherman, '32) in terms of Bourquin-Sherman 'units.' This 'unit' is based on the amount of food required to give a gain in weight of 3 gm. per week or a total gain in body weight of 24 gm. for an 8-week period,

when the Bourquin-Sherman diet is employed. When taken as the gain above the negative controls, 'unit' growth then becomes a gain of $24 + 4 = 28.0$ gm. Referring to the use of the Munsell ration for the determination of vitamin G and to calculation of the resulting data in terms of Bourquin-Sherman 'units,' Sherman ('32) states that "if under other conditions, as in the work described in 1931 by Munsell, the

TABLE 1

The vitamin G (flavin) content of some foods

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD		BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM [1]
			Actual gain	Gain above negative controls	
	gm.		*gm.*	*gm.*	
Cottonseed meal	0.50	6	+ 51.0	+ 41.0	2.92
	1.00	6	+ 85.0	+ 75.0	
Soy beans [2]	0.50	6	+ 55.0	+ 45.0	3.20
Dried whole milk [3]	0.25	6	+ 47.0	+ 37.0	5.28
	0.50	6	+ 83.0	+ 73.0	
Dried brewer's yeast [4]	0.15	6	+ 94.0	+ 84.0	20.00
Negative controls on basal ration	0.00	7	+ 10.0	....	....

[1] One Bourquin-Sherman flavin 'unit' is equivalent to a gain of 28 gm. above the gain of the negative controls.

[2] Biloxi variety.

[3] Made from milk produced in South Carolina.

[4] Used by the South Carolina State Board of Health in combatting pellagra.

weight curve for the negative controls is appreciably different, the numerical 'values' should be corrected accordingly. For this purpose, a unit rate of gain in weight may be taken as 3.5 gm. per week above that of the corresponding negative control animals." On this basis, therefore, a gain of 28 gm. in 8 weeks above the gain of the negative controls represents one Bourquin-Sherman 'unit.' Applying this method of calculation to our data, we have expressed the flavin potency of the foods which we tested in terms of 'units' in table 1.

From a study of the results shown in table 1, it is evident that the foods investigated are all good sources of flavin, the order of decreasing vitamin potency in units per gram being: dried brewer's yeast, 20.0; dried whole milk, 5.28; soy beans, 3.20; and cottonseed meal, 2.92. In calculating the 'unit' values, we have assumed the gain in body weight (above the gain of the negative control rats) to be proportional to the dosage, our results with two levels each of cottonseed meal and of milk justifying this assumption. Where more than one level of the same food was fed, we have used the level yielding the gain nearest 'unit' growth for calculation of the flavin potency. The protocols of our assays also reveal that there was no significant diminution in the rate of growth when the gains in weight (above the negative control gains) were compared at the end of 4 and 8 weeks, indicating that throughout the test period the basal diet plus the supplement furnished an adequate supply of all growth factors for the rates of gain obtained.

In rats that had developed varying degrees of alopecia prior to feeding the supplements, cures of this condition were effected by the various foods tested. By the end of the test period the seven negative control rats, however, developed marked alopecia and other evidences of the 'non-specific' type of skin lesion described by Chick, Copping and Edgar ('35), György ('35 a) and Copping ('36 a, b). At the end of the curative period, the negative controls were continued on the basal ration for an additional 20 days during which time the skin and hair condition were even more characteristic of the 'non-specific' type of lesion. The basal ration was then augmented by the inclusion of 25% cottonseed meal. The animals were fed this ration for a period of 44 days, during which time the average body weight increased from 96 gm. to 206 gm.—a gain of 110 gm. The abnormal skin and hair condition of all the rats was cured and animals regained normal body vigor, demonstrating the ability of the flavin in the cottonseed meal to cure this type of skin disorder. During the period of cottonseed meal feeding, the average daily intake of the meal was 3.0 gm.

In some preliminary experiments designed to concentrate the flavin in cottonseed meal, we prepared a 50% (by weight) ethyl alcohol extract which we fed to one rat that had been kept on the basal ration for a period of 45 days. The extract was fed at a time when the rat was losing weight and exhibited a marked denuded skin condition. The animal was fed 0.3 gm. of the extract solids daily with the result that a gain in body weight of 30 gm. was effected in a period of 32 days, while the abnormal hair condition was completely cured. This preliminary finding demonstrated the presence of flavin in the alcoholic extract of cottonseed meal and prompted us to test the extract again on a larger number of rats.

Series II

The results described in series I led us to carry out further tests employing this time the Bourquin-Sherman flavin-deficient ration. We retested, therefore, the cottonseed meal and brewer's yeast with the Bourquin-Sherman diet in order to ascertain whether the two flavin-deficient rations would yield the same flavin values.

The alcoholic extract of cottonseed meal previously mentioned was also included in this series along with the residue from the extraction in order to determine what proportion of the vitamin was extracted and whether there was any loss in the process which was as follows: Five hundred grams of cottonseed meal[3] were extracted three times with 1000 cc. portions of 50% alcohol for 2 hours at room temperature. The extracts were separated by filtration under suction, combined, centrifuged free from suspended matter, and then evaporated to dryness on a steam bath. A brown sticky residue that became brittle and hard on cooling resulted. Eight such preparations using the same batch of cottonseed meal yielded an average of 66 gm. of extracted solids amounting to 14.2% of the cottonseed meal (dry basis). It was

[3] Upon analysis, the cottonseed meal was found to have the following percentage composition: Moisture, 7.43; fat, 7.45; crude fiber, 14.41; protein, 34.12; ash, 5.75 and N. F. E., 30.84.

determined that the third extraction removed only 8.5% of the extracted solids. The resultant cottonseed meal residue was dried at 60°C. in an electric oven.

The results of the assays are given in table 2. With the lower levels of the supplements, it was noted that not only was there a steady increase in body weight of the rats during the 8 weeks test period, but also that the total gains in weight were almost proportional to the level of the supplement fed.

TABLE 2

The vitamin G (flavin) potency of a) cottonseed meal, b) the 50% alcoholic extract of cottonseed meal, c) cottonseed meal residue and d) dried brewer's yeast

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD	BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM
	gm.		*gm.*	
Cottonseed meal	1.00	6	+ 63.0	
	0.50	8	+ 32.0	
	0.30	8	+ 21.2	2.94
50% alcoholic extract of cottonseed meal	0.40	7	+ 81.0	
	0.30	8	+ 65.0	
	0.20	8	+ 48.0	10.00
Cottonseed meal residue after extraction	1.00	5	+ 48.0	2.00
Dried brewer's yeast	0.20	6	+ 103.0	
	0.10	7	+ 51.5	21.4
Negative controls on basal ration	0.0	7	— 4.0	....

Bourquin and Sherman with whole milk powder and Poe and Gambill ('35 a and b) with tomato juice as supplements likewise secured similar findings where their supplements yielded comparable gains in weight. We also observed that wherever alopecia was present at the start of the curative period, it was cured in every case by the various supplements including the cottonseed meal extract. Such findings indicate that a combination of the basal ration plus the supplements contained an amount of all factors adequate for a rate of gain of at least 3.0 to 6.0 gm. weekly and sufficient to maintain a healthy skin condition in the rat.

As shown in table 2, a level of 0.30 gm. cottonseed meal gave a gain of + 21.2 gm. in body weight which was nearest to 'unit' growth of + 24.0 gm. By calculation at this level, a value of 2.94 Bourquin units per gram is obtained which agrees quite closely with the value obtained in series I. In the case of the solids of the alcoholic extract of cottonseed meal, we have also taken for calculation of potency the level yielding a gain nearest 'unit' growth. On this basis, the extract solids have a potency of 10.00 Bourquin units per gram, while the potency of the cottonseed meal residue was found to be 2.00 units per gram. The dried brewer's yeast was found to contain a potency of 21.4 units per gram which is comparable with the value of 20.0 units secured in series I

TABLE 3

The distribution of vitamin G (flavin) in the various cottonseed meal fractions

FRACTION	WEIGHT OF FRACTION	FLAVIN POTENCY (UNITS PER GRAM)	TOTAL UNITS IN EACH FRACTION	
	gm.			
Cottonseed meal	500[1]	2.94	1470	
Cottonseed meal alcoholic extract solids	66	10.00	660	1454
Cottonseed meal residue	397	2.00	794	

[1] Equivalent to 463 gm. moisture-free cottonseed meal.

with the Munsell ration. The extract solids are therefore 3.4 times as potent as the cottonseed meal, about one-half as potent as the yeast and about twice as potent as dried whole milk. From the results secured in series I and II, it can be concluded that both the Bourquin-Sherman and the Munsell flavin-deficient rations yield the same 'unit' values, when the difference in the body weight curves of the negative control rats on the two basal rations is taken into consideration in calculating the vitamin potency.

A vitamin G (flavin) balance sheet covering the various fractions resulting from the extraction of cottonseed meal was drawn up, yielding the results shown in table 3. From a study of this table, it is revealed that the entire potency of the cottonseed meal could be accounted for, about one-half of

the total flavin units appearing in the alcoholic extract, the remainder in the residue. No loss of flavin occurred during the extraction process.

Series III

In series I and II, the assays were carried out on uncooked cottonseed meal and soy beans. In preparing the soy bean for human consumption, it is customary to cook the bean, so that we felt it advisable to determine whether the cooking process brought about any destruction of flavin. Likewise, we felt

TABLE 4

The influence of pressure cooking on the vitamin G (flavin) potency of cottonseed meal and soy beans

SUPPLEMENT FED	AMOUNT FED DAILY	NUMBER OF RATS	GAIN IN BODY WEIGHT IN THE 8-WEEK CURATIVE PERIOD	BOURQUIN-SHERMAN FLAVIN UNITS PER GRAM
	gm.		*gm.*	
Uncooked cottonseed meal	0.6	8	+ 42.6	2.96
Pressure-cooked cottonseed meal	0.6	8	+ 32.0	2.22
	1.2	8	+ 65.0	
	1.8	8	+ 88.0	
Uncooked soy beans	0.4	8	+ 23.3	2.43
Pressure-cooked soy beans	0.4	8	+ 22.8	2.38
	0.8	8	+ 42.6	
	1.2	8	+ 65.0	
Negative control rats on basal ration	0.0	8	— 4.0	

that if cottonseed meal or flour were to find use as a food, such as an ingredient of bread, biscuits, etc., the effect of heating on the flavin content should be investigated.

Wilkinson and Nelson ('31) observed that when soy beans steamed for 3 hours are incorporated at a level of 10% in a diet devoid of vitamins B and G, normal growth results indicating at least partial stability to heat of the vitamins contained therein. In our experiments both soy beans and cottonseed meal were cooked in an autoclave for 30 minutes at a pressure of 15 pounds. The resulting preparations were assayed using the same feeding technic employed in series II. Both preparations were fed at the levels indicated in table 4.

In each case, the uncooked product was fed at one level to serve as a standard of comparison so that the extent of flavin destruction could be measured.

A study of the table reveals that at a level of 0.6 gm. of cottonseed meal, the uncooked product contained 2.96 units per gram, while the cooked preparation contained 2.22 units indicating that cottonseed meal when cooked as described suffered a loss in vitamin G content of only 25%. On the other hand, 0.4 gm. of soy beans, either cooked or uncooked yielded practically the same potency indicating that no loss of vitamin occurred in the cooking process. On the basis of these tests, therefore, flavin as found in cottonseed meal and in soy beans appears to be quite stable to pressure cooking.

The soy beans (Biloxi variety) used for the cooking tests were not from the same lot as that tested in series I and appear to be only about 76% as potent in flavin as the previous lot. The sample of cottonseed meal which was from the same lot used in series I and II gave practically the same 'unit' value as obtained in the two former series.

Here again, with the various levels of the cooked products, gains in weight practically proportional to the levels fed were secured, testifying to the presence of an adequate content of all growth factors in the combination of the basal diet plus the various supplements.

DISCUSSION

Although several investigators have determined the vitamin G content of milk and yeast, only a few have evaluated the potency of cottonseed meal and soy beans. Drawing his conclusions from indirect experimental data on rats, Stevens ('30) states that commercial cottonseed meal approximates yeast as a source of vitamin G. Essential details are lacking in the report published by this author. On the other hand, however, Sherwood and Halvorsen ('33) have found that cottonseed meal contains about one-eighth as much vitamin G as yeast. On the basis of our assays, cottonseed meal is about one-seventh as potent as the dried brewer's yeast which we

assayed simultaneously. Our findings are therefore quite at variance with those of Stevens and in good agreement with those of Sherwood and Halvorsen. Using the Bourquin-Sherman ration, Whitsitt ('33) fed a group of rats 2.4 gm. of cottonseed meal per week. By calculation using data taken from her growth curves, we find a potency of 3.2 units per gram which agrees closely with our value of 2.92 units. Munsell and Devaney ('33) report a value of 1.7 Bourquin units per gram for cottonseed flour, a finding which suggests that the meal is more potent than the flour and that in refining the meal some flavin is removed. Several investigators (Munsell and Devaney; Sherwood and Halvorsen; Whitsitt; Wilmot and Winters, '34) have reported that cottonseed meal or flour are good sources of vitamin B, one author (Sherwood and Halvorsen) reporting a value of 3 to 4 international units of vitamin B per gram for the meal. On the basis of our findings and the above reports, cottonseed meal can be considered a good source of both vitamin B and flavin.

With respect to the vitamin G potency of soy beans, Wan ('32) found that yellow soy beans contain two-thirds as much vitamin G as whole milk powder. In our assays (series I), soy beans were found to be approximately 60% as potent as dried whole milk, giving good agreement, therefore, with Wan's results. Wan also reports that soy beans contain three times as much vitamin B as dried whole milk. Drake-Law ('32) states that steam-processed soy beans are one-fifth as potent as dried yeast in vitamin G and one-tenth as potent as dried yeast in vitamin B. Since it is known that yeast varies considerably in vitamin B potency, it is impossible to determine from Drake-Law's report whether a high or low vitamin B potency for soy beans is indicated. Wan's finding, however, would indicate that the soy bean is a good source of vitamin B. Soy beans can, therefore, be considered a good source of both vitamin B and flavin.

The vitamin G values for cottonseed meal and soy beans obtained in our assays rank quite high when compared with the values given by Sherman for some common foods.

Sherman reviewing the findings of several workers gives as the vitamin potency of milk a value of 0.40 to 0.75 units per gram on a fresh basis. Recalculated to the dry basis, this value becomes 3.3 to 6.3 units per gram of dried milk. Stiebling and Alleman ('33) report a potency of 5.0 units per gram for skim milk powder; Morgan, Hunt and Squier ('35) a value of 5.6 units for dried whole milk, while Booher and Blodgett ('33) found 0.5 units per gram for fresh whole milk. Our value of 5.28 units per gram for dried whole milk, therefore, comes within the range of potency obtained by these investigators. It is apparent, therefore, that milk produced in a pellagrous area comes within the range of flavin potency of that produced in other regions. The results of our assay show that milk is richer in flavin than cottonseed meal or soy beans. It is about one-fourth as potent as dried brewer's yeast, a finding similar to that reported by Aykroyd and Roscoe ('29).

Munsell and DeVaney report a value of 17.0 vitamin G units per gram for dried brewer's yeast, while Morgan, Hunt and Squier obtained a value of 19.5 units, values which are to be compared with our finding of 20.0 to 21.4 units per gram for our yeast sample. The brewer's yeast used in combatting human pellagra in South Calorina is therefore a potent source of flavin.

Other workers have extracted dried baker's yeast and brewer's yeast employing concentrations of alcohol and other solvents quite close to the concentration that we employed in the extraction of cottonseed meal and have also obtained partial solubility of vitamin G in the solvent yielding results quite similar to ours. Sherman and Sandels ('31) found that 60% (by weight) ethyl alcohol extracted only one-half of the flavin content of dried baker's yeast, while Day ('34) who extracted baker's yeast with various concentrations of either acetone or methyl alcohol in water found that both 60% (by weight) acetone and methyl alcohol extracted only one-half of the flavin content of the yeast. Smith ('33) found about 50% of the potency in the residue when dried brewer's

yeast was extracted with 50% ethyl alcohol (by volume). This partial solubility of the flavin in cottonseed meal and in yeast may be due possibly either a) to concomitant partial insolubility of inorganic salts and protein in the solvent thereby permitting retention of some flavin by adsorption or b) to the existence of the vitamin in the food in both the bound and unbound or free form, only the latter form being soluble in the solvent. György, Kuhn and Wagner-Jauregg ('34) and von Euler and Adler ('34) found that the flavin in yeast is chiefly present in an undialyzable or bound form, combined with a protein carrier as a non-dialyzable 'flavo-protein,' while Supplee and co-workers ('36) suggest that flavin is not merely adsorbed on a substrate, but may be physically or chemically bound.

SUMMARY

1. The Bourquin-Sherman ('31) and Munsell ('31) vitamin G deficient diets were employed to measure the flavin content of various foods. Since pure flavin was unavailable at the time the assays were carried out it was not possible to make parallel feeding tests. The results are, therefore, based on the reliability of technic employed. Hence, the flavin potency of the foods tested is expressed in terms of Bourquin-Sherman units of flavin rather than in terms of weight of flavin.

2. Cottonseed meal, soy beans (Biloxi variety), dried whole milk and dried brewer's yeast were found to be good sources of vitamin G (flavin) and to contain 2.9, 2.4 to 3.2, 5.3 and 20.0 to 21.0 Bourquin-Sherman units of flavin per gram, respectively.

3. Milk from a pellagrous region (South Carolina) is apparently as potent in flavin as that from other localities.

4. Extraction of cottonseed meal with 50% ethyl alcohol (by weight) at room temperature removes only about half of the flavin content. No destruction of flavin occurred during the extraction process as evidenced by the fact that the remainder of the flavin was found in the residue. The solids of the alcoholic extract contained about 10.0 Bourquin-Sherman flavin units per gram representing a vitamin concentration of 3.4

times and a potency about one-half that of dried brewer's yeast and almost twice that of whole milk powder.

5. The flavin as found in soy beans and in cottonseed meal appeared to be quite stable to pressure cooking at 15 pounds for 30 minutes.

6. When differences in the body weight curves of negative control rats are taken into consideration in calculation of the flavin potency of a food, the Munsell and the Bourquin-Sherman flavin-deficient diets yielded almost the same 'unit' values for the foods studied.

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THE MINIMUM VITAMIN A AND CAROTENE REQUIREMENT OF CATTLE, SHEEP AND SWINE [1]

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A previous paper (Guilbert and Hart, '35) presented data on the carotene requirement of cattle. From these data, together with available information on other species, the generalization was made that vitamin A requirement is directly related to body weight rather than to energy requirement and that the minimum to prevent or cure deficiency symptoms and to permit normal growth of mammals was in the order of 20 to 30 micrograms daily per kilogram body weight. Before that paper was offered for publication, confirmatory evidence had been obtained with sheep and with swine; that is, doses of alfalfa meal, calculated on the basis of the generalization given above, the weight of the individuals, and the carotene analysis of the feed, uniformly cured the symptoms of deficiency. We now present data on the requirement of swine and sheep in terms of vitamin A as supplied by cod liver oil, in addition to carotene as furnished by alfalfa and solutions of the crystals in cottonseed oil. Experiments on the vitamin A requirement of cattle, using cod liver oil as the source, are also reported. These data are considered in relation to similar information on the rat.

[1] This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

METHODS OF PROCEDURE

Carotene analysis of feeds. The method of carotene analysis employed was a slight modification of the procedure proposed by Guilbert ('34). Briefly the procedure consists of subjecting the sample to saponification in aldehyde-free alcoholic KOH, and extraction with peroxide-free ethyl ether. The ether solution, after washing free from chlorophyllines, flavones and alkali, is distilled under reduced pressure, the residue taken up in petroleum ether and the xanthophyll removed by washing with 85 to 90% methanol. The resulting solution of carotene in petroleum ether, after making up to convenient volume is determined colorimetrically, using for comparison a dye solution prepared from orange G and naphthol yellow crystals. The dye solution was standardized against two samples of beta carotene having melting points of 182°C. and 184°C., respectively, that were prepared in the laboratory of the Carnegie Institution of Washington, Division of Plant Biology at Stanford University and supplied to us through the courtesy of Dr. H. H. Strain. Since no more carotene is extracted from the samples by other appropriate solvents nor by other procedures, the extraction is considered to be quantitative. Numerous determinations run with beta carotene, M.P. 182°C., and with the beta carotene added to alfalfa samples, have resulted in recovery of 95 to 98% of the added carotene when subjected to the routine procedure. Close agreement has been obtained on samples analyzed by various research and commercial laboratories that are using this method. That the biological response is proportional to the value found by carotene analysis was found through trials with alfalfa meals varying over tenfold in carotene content (Guilbert and Hart, '35).

Analysis of cod liver oil. A 30 gallon drum of medicinal grade cod liver oil, guaranteed to contain not less than 2100 U.S.P. 1934 revised units per gram, was obtained for these experiments. Analyses were run on dilutions of this oil and upon the unsaponifiable fraction with a Hilger Vitameter-A, equipped with a photographic attachment. The method of

saponification and preparation of the sample followed was that recommended in the report of the second conference on vitamin standardization (League of Nations, Quarterly Bulletin of the Health Organization, vol. 3, no. 3, September, 1934). The calculation of percentage vitamin A is based upon the value $E^{1\%}_{1\,cm.}$ 328 mμ = 1600 (Carr and Jewell, '33). Determinations on this oil at the beginning and at the end of the experiments showed no change in its extinction coefficient. The value found by this procedure was 0.083% vitamin A (Carr and Jewell concentrate). The 1934 conference on vitamin standardization recommended the factor 1600 for converting values obtained for $E^{1\%}_{1\,cm.}$ 328 mμ into a figure representing international units of vitamin A per gram of the material tested. On the basis of this calculation the cod liver oil contained 2120 units per gram, a value in agreement with the producer's guaranteed minimum.

Similar Vitameter-A analyses of U.S.P. reference cod liver oil which is assigned a value of 3000 units per gram, gave a value of 2070 biological units. This result is similar to the average value obtained with this instrument by eight collaborators and reported by Irish ('36). The discrepency in these results (aside from possible inherent limitations of the instrument) indicate that either the conversion factor or the U.S.P. reference cod liver oil is in error. Since this question is at present controversial (Bacharach, Drummond and Morton, '36) calibration of the Vitameter on the basis of the U.S.P. reference cod liver oil did not appear justified. We have, accordingly, used the value 0.083% vitamin A (Carr and Jewell concentrate) in the calculations of minimum vitamin A requirements reported in this paper, recognizing, however, that this value is subject to revision.

Very little difference was found in the Vitameter values obtained with dilutions of the oil used in these experiments or of the U.S.P. reference oil compared with their unsaponifiable fractions. Furthermore, the values found by the antimony trichloride reaction were roughly proportional to their respective Vitameter-A values. These results indicate that both

oils were comparatively free from interferring irrelevant material.

A quantity of oil from the batch used in these experiments has been saturated with CO_2 and stored at low temperature, thus providing a reference for reinterpreting the biological results, if within a reasonable time, a more satisfactory basis for evaluation becomes available.

Criteria used to determine minimum requirements. Investigations with cattle (Guilbert and Hart, '35) showed that night blindness was the first detectable symptom of deficiency to appear on a deficient ration and the last to disappear as the dosage of carotene was increased from sub-minimum to minimum levels. A level of carotene intake that just prevented night blindness sufficed also for excellent gains; the animals remained in thrifty condition for indefinite periods, though their storage was meager. Preliminary investigations with swine and with sheep demonstrated that the night blindness test applied equally well with these species, although occasionally, swine developed partial posterior paralysis before defective vision in semi-darkness had been demonstrated. The sheep, in our experiment, developed a partial optical impairment of a permanent nature in a shorter time after the appearance of night blindness than did cattle or swine. This eventually rendered half of our original number of sheep useless for the study of minimum requirement by means of the night blindness test. Numerous animals, particularly pigs, were autopsied soon after showing defective vision in semi-darkness. The unsaponifiable matter from 20 gm. of the liver tissue in these cases, concentrated to 2 cc. or less in chloroform solution, either failed to give any test with antimony trichloride or gave only a trace of blue color, thus showing that vitamin A reserves were practically exhausted. In the experiments with sheep and with cattle in which the number of animals was limited, the doses of vitamin A or of carotene were repeatedly increased and decreased until within reasonably narrow limits the least amount that prevented night blindness was found. Subsequently this level of dosage was held constant with

reference to body weight for periods varying from a few weeks to several months, and the amount of storage checked either by the time required for symptoms to reappear after the supplement was discontinued or by slaughtering the animals and determining the extent of storage in the liver by the antimony trichloride method. Night blindness consistently reappeared in such animals within 1 to 3 weeks after vitamin A therapy ceased; those slaughtered while receiving the minimum level had little storage as shown by the antimony trichloride reaction. While this procedure was also employed with swine, most of the data were obtained by placing different groups of animals on varying levels of vitamin A or carotene intake and maintaining these levels constant with reference to body weight throughout the experimental period. Examination of the liver extract by the antimony trichloride technic was made for all animals that died and for those slaughtered at the close of each experiment.

Practically normal gains, over long periods of time, have been made by animals receiving vitamin A or carotene in amounts that did not permit normal vision in semi-darkness. Apparently only when sufficient vitamin A is provided to meet the needs of all of the body tissues is there sufficient available to form adequate amounts of the visual pigments as elucidated by Wald ('34). Night blindness appears as the first evidence of want and the rapidity with which it may be cured is evidence that recovery is not complicated by repair of pathological tissue. The minimum intake to prevent it appears to represent a true physiological minimum, since the animals may make excellent gains and continue in a state of thrift for many months. The test applies equally well with young and with mature animals and to the different species under investigation. It is apparently conditioned upon a single physiological function of vitamin A, that is, the visual pigment cycle, as compared with the many complications of growth. The test in our experience is sufficiently sensitive so that the effect of varying the dosage of vitamin A over a range of 25% is consistently detected. It is relatively unaffected by such

factors as heredity, age, energy intake, and environmental factors that must be most rigidly controlled to assay within similar limits the vitamin A value of feeds by the growth method. The animals were tested by driving them about their lots in twilight, moonlight, or in dim electric light. Animals frequently show a remarkably memory of their surroundings and may run about quite freely even though sight is very defective. To avoid this error, barriers were placed in different locations to form a maze. By this means defective animals were readily detected and different degrees of night blindness noted. Swine also frequently demonstrated defective vision by being very difficult to drive, while normal animals moved about freely. The night blindness tests were made at least once and more often twice weekly and the observations recorded by four independent observers.

Rations used and method of feeding. The concentrate rations used are given in table 1, together with the total carotenoid pigment in the rations that was not extracted from petroleum ether solution by 90% methanol. Most of the pigment in the petroleum ether fractions of the swine rations, when passed through adsorption columns composed of equal parts of MgO and siliceous earth (method of Strain, '34), adsorbed strongly at the top of the column. A small portion separated and washed through quite readily, thus indicating that not all of the pigment was beta carotene. H. H. Strain (personal communication) has, as a result of his studies of the carotenoids of barley grain, confirmed our assumption that most of the petroleum ether fraction was beta carotene. Markley and Bailey ('35) have shown that the pigment remaining in the petroleum ether fraction from wheat grain is largely carotene. The colorimetric value that we found for barley grain is in good agreement with that calculated from biological tests with rats (Hughes, '33). The analyses indicate, therefore, the maximum contribution of vitamin A value from the basal rations and that no serious error results from considering the pigment as beta carotene for the purpose of these experiments.

For roughage in the cattle ration, wheat or barley straw was used. It had stood several weeks in the field after ripening before it was harvested, and contained no trace of green color. The extract from 100 gm. concentrated to small volume contained only a trace of yellow pigment. No yellow pigment was extracted by ether and alcohol from the cottonseed hulls that were used as roughage in the sheep rations. Since the

TABLE 1

Basal concentrate rations and their approximate carotene contents

COMPONENTS OF RATION	CATTLE	SHEEP	SWINE		
			No. 1	No. 2	No. 3
Dried molasses beet pulp	70.0	50.0			
Rolled barley	14.0	10.0	73.0	34.0	12.0
Cottonseed meal	15.0	30.0			
Brewers' rice				30.0	58.0
Wheat middlings			15.0	14.0	20.0
Wheat bran		10.0			
Tankage			10.0		8.0
Linseed meal				5.0	
Skim milk powder				15.0	
$CaCO_3$	1.0		1.0	1.0	1.0
NaCl	Ad lib.	Ad lib.	1.0	1.0	1.0
Carotene content milligram per cent[1]	< 0.02	< 0.02	0.02	0.02[2]	0.023

[1] These values represent the total pigment remaining in the petroleum ether fraction after extraction with 90% methanol and, as discussed in the text, is not all beta carotene.

[2] This ration, after substituting new for old crop barley, contained 0.067 mg.% 'carotene.'

concentrate rations were limited for cattle and sheep not to exceed 1.5 pounds daily per 100 pound body weight, the amount of carotene contributed by the basal rations was relatively insignificant and no correction for it has been made in the calculations. The swine rations, on the other hand, being higher in carotene and consumed in larger quantities per unit of body weight, contributed significantly toward the animals' requirements. The amounts furnished by the basal

rations daily per unit of body weight were, therefore, calculated and included in the total intake reported for the various groups used in studying the minimum requirement of swine.

The cattle were fed individually in feeding stalls. The dosage of cod liver oil was measured by a glass hypodermic syringe, mixed with a small portion of the concentrate ration and placed on top of the remainder of the concentrate allowance where it would be consumed first.

The sheep were fed in individual feeding stalls except nine head that for a time were fed as a group. Alfalfa supplement was fed by mixing the meal with the concentrate ration once daily. Both cod liver oil and solutions of carotene in oil were administered by means of glass hypodermic syringes graduated to 0.1 cc. that delivered 0.92 gm. of oil per cubic centimeter at room temperature. For lower levels of dosage of cod liver oil it was diluted with cottonseed oil. The doses of oil and dilutions thereof varied from 0.5 to 2.5 cc. daily. After the desired amount of oil had been drawn into the syringe, the outside was wiped clean with a cloth. The animal's head was drawn up, the mouth forced open and the dose deposited on the back of the tongue, the syringe tip being touched to the tongue to remove the adhering drop. As the animals made no attempt to eject the oil, consumption was considered to be quantitative.

Each group of pigs had access to a supply of basal ration in self-feeders and feed records were kept on each group. Alfalfa meal supplements were mixed with a small amount of basal ration, the whole moistened to minimize loss, and fed to the pigs individually in feeding stalls. Cod liver oil and carotene solutions were administered in a manner similar to that described for sheep.

The alfalfa meal used during most of the experiments was a dehydrated product that had been in storage for nearly a year. The carotene content of this dropped from 12.9 to 7.8 mg.% carotene during the course of the experiments. The meal used in the last experiment with swine varied from 17.6 to 18.7 mg.% carotene. The daily allowance for each individual

was weighed into paper bags at weekly intervals. The meal was sampled while the feeds were being weighed; and the composite sample, kept in a tightly covered container at —5° to —10°C., was analyzed for carotene at monthly intervals.

The crystalline carotene was obtained from the S. M. A. Corporation. The melting point was 165° to 168°C. Sufficient amounts for 1- and 2-week periods were dissolved in cottonseed oil and made up to volume so that doses varying from 1 to 2 cc. daily carried the desired amount of carotene. The solutions were analyzed colorimetrically for carotene, and doses calculated on this basis. Repeated colorimetric analyses of aliquots of 100 mg. samples of the carotene, dissolved in cottonseed oil and diluted with petroleum ether (usually 1 to 100), consistently gave a value of 90 to 94% of the quantity weighed, indicating an average of about 8% impurities. Adsorption and separation of the carotene from petroleum ether solutions on magnesium oxide, with subsequent elution and colorimetric determination of the fractions, showed that approximately 88% of the carotene was the beta form. To prevent deterioration, the cod liver oil and carotene solutions were kept in the dark at about 34°F., except at feeding time.

The sheep and swine were weighed at weekly intervals and the cattle were weighed fortnightly. The desired level of vitamin A supplement dosage was maintained practically constant with reference to body weight by adjusting it at each weighing period for any change in weight of the animals.

Cattle used and experimental procedure. Five mature cows that had been on vitamin A experiments for over 3 years were used in the experiments on minimum requirement of vitamin A supplied by cod liver oil. Their vitamin A intake had been controlled so that they could be depleted to the point of night blindness within a short period of time. One of these cows, a negative control, received no supplement of cod liver oil. In addition, a calf, 5 months old at the beginning of the experiment was used. This calf became night blind

while nursing its vitamin A deficient mother. As soon as the animals exhibited night blindness, varying doses of cod liver oil were administered until the approximate minimum level was found. This level of dosage was then kept constant relative to body weight for periods of 2 to 6 months. All of the cows were pregnant during the experimental period. Comments on storage and reproduction will be found in the discussion of results.

Sheep experiments. Depletion periods and procedure. Twenty-one head of 7-year-old ewes were used in a preliminary experiment to study their storage and rate of depletion of that storage on the basal ration. These animals previously had had favorable conditions for storage. Animals were slaughtered at the beginning and at intervals throughout the depletion period and the liver storage determined by the antimony trichloride technic. The initial storage varied from 2500 to 4000 blue units[2] per gram of liver. The liver storage of animals, slaughtered at bi-monthly intervals, declined as the depletion period progressed so that after 10 to 16 months of depletion, the liver values ranged from 200 to 600 blue units per gram of liver. Four remaining animals developed night blindness after 22 to 23 months on the basal ration. These died or were killed in cachexia after 27 to 30 months at which time no vitamin A could be demonstrated in their livers. The withdrawal of vitamin A from storage appeared more rapid during the first year than during the second, a finding analogous to that in cattle. The change in these animals from the onset of night blindness to death was very gradual and was chiefly characterized by anorexia, gradual loss of condition and muscular weakness. In the final stages, the corneas of two of the animals were partially clouded, but

[2] The antimony trichloride 'blue unit' is defined as the amount of vitamin A in 0.05 to 0.2 cc. of chloroform solution that will produce with 2 cc. of antimony trichloride reagent a faint blue color that approximately matches that produced by 1 microgram of carotene under similar conditions. The color also matches a copper sulfate solution equivalent to 0.5 mg. of copper per cubic centimeter. This is used as a standard for comparison. One blue unit on this basis approximates 0.25 international or U. S. P. unit.

sight in daylight was never seriously impaired. Pneumonia, enteritis and kidney involvement were the most conspicuous autopsy findings.

On July 25, 1934, thirty head of ewe lambs were placed in a bare lot and started on the basal concentrate ration and cottonseed hulls. One lamb, slaughtered at the beginning of the experiment, had 750 blue units of vitamin A per gram of liver, whereas two that were slaughtered after 3 and 7 months on the deficient ration, had liver concentrations of 1000 and 250 blue units of vitamin A per gram of liver, respectively. Similar lambs, slaughtered after corresponding periods on green pasture, had 1250 and 3300 blue units, respectively. The liver concentration of vitamin A, therefore, apparently trebled during 7 months on green pasture and reached a value approximating that of the aged ewes.

A ram was placed with the ewe lambs on October 26th and remained until December 1st. Eight of these females conceived. Night blindness first appeared in three of them on March 21, 1935, after 8 months on the deficient ration; and all twenty-seven of those remaining showed night blindness within the next month. Lambing coincided with this period. Of the eight lambs born, seven were weak and either died within a few hours without nursing, or, if they were able to nurse, died within 5 days, one lamb showing severe diarrhea. The liver extract of five of the lambs gave no color test with antimony trichloride, while two gave a trace of color. One lamb was strong and active at birth and was thrifty at 14 days when it was killed. Its liver contained 5 blue units of vitamin A per gram. The mother was one of the last to show symptoms of deficiency. Autopsy findings on the lambs were characterized by general enteritis and more or less congestion in the lungs. All the ewes appeared to have an abundance of milk.

The first eighteen ewes to develop symptoms were started on minimum requirement experiments, using the procedure outlined in the section on criteria used to determine minimum requirements. The last nine to become night blind were

turned onto green pasture, and all were normal at the end of a week, when their vision was tested in moonlight. Data on storage and reproductive history at the close of the recovery experiments are mentioned in the discussion of results. The time for depletion of storage in sheep was similar to that of cattle that had comparable vitamin A concentration in the liver as shown by the antimony trichloride test on animals slaughtered at the beginning of the depletion period.

Swine experiments. Over 100 head of Poland China and Duroc Jersey pigs farrowed in 1934 and 1935 were selected at weaning time, placed in paved lots and used in three sets of experiments involving basal rations 1, 2 and 3, respectively. In addition to these rations a small group of nearly mature hogs was fed for a short period on brewer's rice, a feed extremely low in carotene, supplemented by varying levels of cod liver oil.

Pigs, that received with their mothers, a normal ration including pasture until weaning at about 8 weeks of age required an average period of 146 days on basal ration no. 1 to become depleted. At this time the average weight was 167 pounds. Pigs whose mothers were restricted during lactation to a carotene low ration supplemented with 5% of fair quality chopped alfalfa hay, required from 70 to 120 days to become depleted on basal rations 2 and 3, respectively. Their average weight was about 85 pounds when symptoms of deficiency developed. Most of the pigs were started in the recovery experiments when they had shown night blindness on two or three successive nights. In some cases they were in more advanced stages involving diarrhea and varying degrees of muscular incoordination or partial paralysis. Although the response of these animals to vitamin A or carotene therapy was spectacular, recovery from partial posterior paralysis was usually incomplete.

Fifteen animals in prophylactic experiments involving different levels of carotene furnished by alfalfa meal confirmed the results of the recovery experiments as shown by the liver storage found at autopsy. The effect of a single 10-gm. dose

of halibut liver oil given by stomach tube was studied in four animals. One of these was autopsied after 48 hours. Vitamin A was demonstrated by the antimony trichloride test in the liver, kidney, lean muscle, abdominal fat and blood serum. The recovery was estimated at about 8% of the total ingested. The three remaining pigs grew normally and when they had attained a weight of about 200 pounds, 5 months later, still had a small reserve of vitamin A in their livers.

Fifteen control animals died or were killed in the early stages of deficiency. No vitamin A could be demonstrated in their liver tissues by the antimony trichloride test. During the course of the depletion periods a number of animals died and others were discarded because of unthriftiness. The storage of vitamin A in the livers showed that factors other than vitamin A deficiency were involved in these cases. Only animals that were thrifty during the depletion period were used. Data on fifty of these during the recovery experiments are summarized along with the data for cattle and sheep in tables 2 and 3.

RESULTS

To conserve space the records of individual animals and groups in the various experiments are all combined and presented in summary form in tables 2 and 3.

Table 2 presents the data on carotene requirement when the carotene was furnished by alfalfa meal and by crystalline carotene dissolved in cottonseed oil. The data for cattle from previous experiments are included for comparison. The results with the different species and with the two sources of carotene were practically the same. Twenty-five micrograms of carotene per kilogram body weight appears to be the minimum level that will meet the criteria employed. The data were obtained from mature animals that were maintaining their weight, from pregnant females and from rapidly developing young animals. No detectable difference in the requirement for these varied conditions was evident. Pregnant, non-lactating, cows maintained on minimum levels throughout

gestation, however, have uniformly given birth to weak calves that died soon thereafter. Increasing the carotene intake to three or four times the minimum during the last month of gestation has consistently resulted in normal calves at birth.

TABLE 2

Minimum carotene requirement of cattle, sheep and swine when supplied by alfalfa meal and by the crystals dissolved in cottonseed oil

SPECIES	NUMBER OF ANIMALS	SOURCE OF CAROTENE	CAROTENE INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT	RESULTS
Cattle[1]	10	Alfalfa	< 26	Remained night blind or partially defective
Sheep	14	Alfalfa	9 to 24	Two animals recovered on highest level, remainder continued night blind
Sheep	3	Carotene in oil	18 to 26	Remained night blind or partially defective
Swine	11	Alfalfa	16 to 22	Remained night blind or partially defective. Highest dosage borderline. Liver storage, negative to trace
Swine	2	Carotene in oil	19 to 20	Remained night blind, died, no storage in livers
Cattle[1]	10	Alfalfa	26 to 33	Recovered, small storage in liver, gains normal
Sheep	11	Alfalfa	25 to 35	Recovered, small storage in liver, gains normal
Sheep	3	Carotene in oil	27 to 36	Recovered, remained normal for 2 months. Symptoms recurred within a week after supplement was stopped
Swine	20	Alfalfa	25 to 39	Recovered, small storage in liver, gains normal
Swine	4	Carotene in oil	26 to 33	Recovered, remained normal for 3 months, small storage in liver

[1] Data from Guilbert and Hart ('35).

Similar treatment of sheep at the end of gestation in the present experiments did not result in any normal lambs. The results are complicated, however, by the fact that all of the ewes had manifested deficiency symptoms at various times

during pregnancy. No vitamin A could be demonstrated in the livers of any of the dead lambs.

Cows that had practically no reserve of vitamin A have been maintained for long periods during lactation on approximately the minimum level of carotene intake without developing any clinical symptoms of deficiency. The milk in these cases was practically devoid of vitamin A and nursing calves developed symptoms and died. Milk production was adequate to promote fair growth in the calves when they received a vitamin A supplement. In one case (a Shorthorn beef cow) in which milk and butter fat production records were kept, the milk production was not increased as the carotene intake was increased from the minimum level to prevent night blindness in the cow (15 mg. daily) to an intake of over 1 gm. daily, an amount in excess of that necessary to produce the maximum carotene content of butter fat for this animal. The butter fat production averaged about 0.5 pound daily.

Six sows that received slightly sub-minimum amounts of alfalfa supplement for several months, came into oestrum and mated more or less regularly, but no litters were produced.

Table 3 summarizes the results with cod liver oil. Although the results in terms of absolute quantities of vitamin A are subject to revision, they are, as with carotene, consistent for the different individuals and for the species under investigation. On the basis of the analysis employed, 6 to 8 micrograms daily per kilogram body weight were the least amounts that met the criteria for minimum requirements. In terms of the cod liver oil used, this represented doses of 0.36 to 0.48 cc. for each 100 pounds of body weight. Slight excesses appeared to result in more rapid accumulation of liver storage than was found under similar conditions with carotene.

Two non-lactating cows that were maintained on the minimum level of intake throughout most of gestation, were given between three and four times this amount during the last month. Both produced normal calves that remained thrifty and grew normally for 2 months while receiving their mother's milk. A third cow that had a prolapsed vagina 7 days before

normal calving date, was sacrificed and the calf delivered by Caesarian section. The calf was strong, active and appeared normal in every respect. Its liver contained 5 blue units of vitamin A per gram, a meager amount but not unusual in the newborn. Two cows were allowed to become deficient and developed symptoms during the last month of gestation. One aborted a 3-week premature fetus; the other had at term a weak calf that died the second day. The results with regard to reproduction are similar to those with carotene, except

TABLE 3

Minimum vitamin A requirement of cattle, sheep and swine using cod liver oil as the source and based on Hilger Vitameter-A analysis of the oil

SPECIES	NUMBER OF ANIMALS	VITAMIN A INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT	RESULTS
Cattle	2	4.3 to 5.6	Remained night blind, no improvement in 3 weeks
Sheep	5	3.1 to 4.1	Developed night blindness while receiving these amounts
Swine	3	3.7 to 4.4	Remained night blind for 2½ months. Gains nearly normal
Cattle	5	6.7 to 8.4	Recovered. Remained normal for 2 to 6 months. Small storage in liver
Sheep	5	5.6 to 8.2	Recovered. Gains normal. Small storage on higher level
Swine	8	5.8 to 7.5	Recovered. Gains normal. Small storage in liver

like levels of carotene with respect to the minimum has resulted in deficiency in the calves within the first 3 weeks of the nursing period. This fact also indicates a somewhat more efficient utilization of preformed vitamin than of carotene.

Four ewes that received approximately three times the minimum level of cod liver oil during the last 2 to 3 weeks of gestation produced dead lambs or lambs that died within a few hours. No vitamin A could be demonstrated in the livers of the lambs by the antimony trichloride test. All of the ewes had shown symptoms of deficiency at various times during gestation, when the rate of dosage was being varied to determine minimum requirements.

All of the pigs were carried on the levels indicated in table 3 from the time of development of symptoms until they reached market weight (about 200 pounds). The average time was about 2½ months. The gains of those on the sub-minimum levels average 1.25 pounds daily; those on approximately minimum levels averaged 1.47 pounds daily, a very satisfactory gain for such animals. The liver storage of the latter varied from 3 to 12 blue units per gram, which represents a very small reserve.

TABLE 4

Summary of data on the minimum requirement of cattle, sheep and swine for vitamin A and carotene compared with the biological unit and other data for the rat

SPECIES	SOURCE OF DATA	DAILY INTAKE, MICROGRAMS PER KILOGRAM BODY WEIGHT	
		Vitamin A	Carotene
Cattle	Author's data	6.7–8.4	26–33
Sheep	Author's data	5.6–8.2	25–35
Swine	Author's data	5.8–7.5	25–39
Rat	Bauman et al. ('34)	.....	25
Rat	Biological unit[1]	4	6

[1] Based on the conversion factors recommended by the 1934 conference on vitamin standardization and the estimation that the rats average 100 gm. in weight during the test period.

DISCUSSION

In order to compare the information secured with cattle, sheep and swine with data on the rat and in the light of these figures to examine the hypothesis previously proposed that vitamin A requirement is directly related to body weight, table 4 is presented.

The 1934 conference on vitamin standardization defined the international unit as the vitamin A activity of 0.6 microgram of pure beta carotene. As a result of the comparison of thirty-five biological assays on twelve different materials with percentages of vitamin A determined spectrographically, 1 gm. of the Carr-Jewell concentrate, which is assumed to be nearly pure vitamin A, was assigned a value of 2.56×10^6 international units. One unit on this basis, therefore, equals 0.4 microgram of vitamin A (Carr-Jewell concentrate). These

values have been converted to the basis used for expressing the requirement of domestic animals by assuming that the rats used in biological assays average 100 gm. in weight during the test period.

It was further stated in the report on vitamin standardization "that daily doses of 2 to 4 international units of vitamin A when administered to young rats suitably prepared on a vitamin A-deficient diet, have been found adequate to restore growth." Twice the vitamin A biological unit dosage shown in table 4 coincides with the minimum found for domestic animals on the basis of the Hilgar Vitameter-A analysis of the oil. Four times the biological unit of carotene is comparable with the minimum that we found for carotene. Although this means of comparison is, for numerous reasons, open to criticism, the apparent agreement, nevertheless, appears to be significant. The data on carotene requirement is more direct and definite. Bauman, Riising and Steenbock ('34) found that 10 micrograms of carotene per week permitted moderate growth in rats while doses in excess of 20 micrograms per week did not enhance growth. Since their animals averaged about 115 gm. in weight during the experimental period, the average daily intake at the latter level was about 25 micrograms per kilogram body weight, a value that coincides with our data. The minimum requirement for carotene is also within the limits found for swine by Dunlop ('34). He states: "The vitamin A requirement of swine has been shown to be between 14 and 62 mg. of carotene per 100 pounds of ration." By calculation from the feed consumed and weights of animals in his experiments these limits represent an intake of 13 to 60 micrograms daily per kilogram body weight. The lower level supported nearly normal growth but deficiency symptoms persisted; on the higher level considerable storage occurred.

It has already been pointed out (Guilbert and Hart, '35) that the energy requirement per unit of weight of 100 gm. rats must be at least eight times that of adult cattle. Direct relation of vitamin A to energy requirement is, therefore,

contra-indicated. The agreement between species and between animals of the same species differing greatly in body weight leads to the conclusion that vitamin A requirement is directly proportional to body weight or to the weight of tissues that are highly correlated with body weight.

Vitamin A is known to be associated with nerve tissue and with epithelial structures throughout the body. It is involved in the visual process (Wald, '34). That vitamin A acts as an oxidizing catalyst has been used as a working hypothesis in some experiments (Euler, '31). It has been reported by numerous workers to be interrelated with thyroxine. It appears, therefore, that vitamin A is involved in the normal functioning of a variety of tissues and structures. The data on minimum requirement suggests that its function in the normal animal is related to the amount of these tissues rather than to their rate of metabolism.

Having been able to predict the carotene requirement of sheep and of swine on the basis of the generalization derived from the data on cattle and rats, and having found that the vitamin A requirement is also uniform with reference to body weight in all of these species, we are impressed with the possibility of extending the information to other mammals. Calculations from the clinical data of Hess, Lewis and Barenberg ('33) and from those cited by Garry and Stiven ('36) indicate that the human requirement for carotene and for vitamin A cannot be far different from that of the species studied. The calculations for the former were based upon the values for milk reported by Semb, Bauman and Steenbock ('34) and by Gillam ('34); for the calculations from later data, present conversion factors for biological units were used. A possible limitation of the prediction that the vitamin A requirement of mammalia is 6 to 8 micrograms per kilogram (on the basis of present criteria) and the carotene requirement 25 to 30 micrograms daily per kilogram body weight may exist in the carnivores that in nature are accustomed to receive this factor as preformed vitamin A. Some evidence in this direction is given by Frohring ('35) who found that considerably larger

doses per unit of weight of carotene were required to cure deficiency in puppies than in rats.

Although the requirements in terms of absolute quantities of vitamin A are subject to revision, it appears from the data presented that at low levels of intake, vitamin A and carotene approach biological equivalence, and that the ratio of their respective efficiencies widens as the dosage is increased. Davies and Moore ('34) and others have shown that much higher percentages of large doses of vitamin A are assimilated and stored than of carotene.

From the studies of storage at different levels of intake in animals it appears that five to ten times the amount necessary to satisfy our definition of the minimal intake, is a desirable minimum to set for practical purposes. Significant storage accumulates within a few months at such a rate of intake and a reasonable storage reserve is desirable. Even this factor of safety, however, results in the production of milk low in vitamin A value, at least when its source in the ration is carotene and when the reserves of the animal are low.

Our experience with different species of animals shows that, although the minimum requirement per unit of body weight is the same for young and old animals, the former are more susceptible to pathological manifestations during vitamin A privation. The young animal is particularly susceptible to severe diarrhea and while it almost invariably occurs in older animals the condition does not ordinarily become serious. When diarrhea is caused by the lack of this factor the recovery following vitamin A therapy is spectacular. Young animals are likewise more susceptible to nerve injury. Whether or not the apparently greater resistance of older animals to pathological changes in the tissues is due to inherent differences in the tissues or to the slow withdrawal of the last vestiges of reserves from adipose tissues must be left open.

SUMMARY

The results of experiments on the minimum vitamin A and carotene requirement of cattle, sheep and swine are reported. The night blindness test, supplemented by a check on storage either by depletion or by the antimony trichloride test on extracts of the liver tissue, was used as the criterion of sufficiency. Evidence was presented that the amount of vitamin A or carotene daily that just prevents night blindness, represents a physiological minimum. Carotene was furnished by alfalfa and by crystalline carotene dissolved in cottonseed oil. Vitamin A was supplied by cod liver oil. It was analyzed with a Hilger-Vitameter A and also by the antimony trichloride method and the results compared with similar analysis of U. S. P. reference cod liver oil. Limitations of expressing vitamin A in absolute terms are discussed.

The minimum carotene requirement for all of the species studied was found to be 25 to 30 micrograms daily per kilogram body weight, an amount in agreement with similar data on the rat. The minimum vitamin A requirement on the basis of the analysis and criteria used was found to be 6 to 8 micrograms daily per kilogram body weight. These figures also appear to agree well with data on the rat calculated from present conversion factors for biological units. Excellent growth occurred at these levels yet storage after extended periods was meager. The data support the hypothesis proposed by Guilbert and Hart ('35) that vitamin A requirement is directly related to body weight rather than to energy requirement and that the requirement of other species of mammals may be predicted on this basis. Calculations from clinical data indicate that the human requirement cannot be far different from that of the species studied. Indications of poor use of carotene by puppies is cited as a possible limitation in applying the generalization for carotene to carnivores.

Data on depletion periods for swine and sheep together with observations on reproduction are presented and discussed.

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